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(54) Title: TUMOR NECROSIS FACTOR ALPHA CONVERTASE (57) Abstract <p>The present invention relates to tumor necrosis factor alpha (TNFα), and more specifically to the enzyme TNFα-convertase (TNFα-con) that can proteolytically convert TNFα precursor to mature TNFα. The present invention provides DNA sequences encoding mammalian TNFα-con and functional equivalents thereof, recombinant expression vectors comprising said DNA sequences, host cell lines comprising said expression vectors, inhibitors of TNFα-con, inhibitors modified for use as ligands for affinity purification of TNFα-con, and methods for treating diseases or conditions resulting from abnormal levels of TNFα in a mammalian subject.</p>		

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TUMOR NECROSIS FACTOR ALPHA CONVERTASE

1. FIELD OF THE INVENTION

The present invention relates to tumor necrosis factor alpha (TNF α), and more specifically to the enzyme TNF α convertase (TNF α -con) that can proteolytically convert TNF α precursor to mature TNF α . The present invention provides DNA sequences encoding mammalian TNF α -con and functional equivalents thereof, recombinant expression vectors and host cells comprising said DNA sequences, methods for making TNF α -con using such DNA sequences, inhibitors of TNF α -con, inhibitors modified for use as ligands for affinity purification of TNF α -con, and methods for treating diseases or conditions resulting from abnormal levels of TNF α in a mammalian subject.

The invention is demonstrated by a working example in which TNF α -con is isolated and a cDNA encoding human TNF α -con is cloned and sequenced.

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2. BACKGROUND OF THE INVENTION

TNF α , also known as cachectin, is a mammalian protein that is produced primarily by activated monocytes and macrophages. TNF α is a potent cytokine that plays a pivotal role in host defense against invasion by microorganisms by mediating cellular responses to infection. TNF α is generally not present in measurable amounts in normal mammalian sera, but appears rapidly in response to several types of stimuli, including infection by viruses or bacteria, trypanosoma and plasmodia, and the cytokine IL-1 (Beutler and Cerami, 1989, Ann. Rev. Immunol. 7:625-655). The most potent known stimulus of TNF α production is bacterial lipopolysaccharide (LPS).

TNF α is an important endogenous factor in the pathogenesis of septic shock (Williams and Summers, 1994, Exp. Opin. Invest. Drugs 3:1051-1056), and in chronic wasting (cachexia) associated with acute inflammatory or malignant diseases (Vassali, 1992, Ann. Rev. Immunol. 10:411-452;

Beutler and Cerami, 1989, above). TNF α has been recognized as manifesting a dose dependent toxicity. For example, if TNF α is present at high levels even for a short period of time, it may trigger septic shock. If TNF α is present at low levels for too long a period of time, it may result in cachexia.

Abnormal levels of TNF α have also been implicated in the pathogenesis of the following diseases or conditions: systemic inflammatory response syndrome, reperfusion injury, cardiovascular disease, infectious disease, obstetrical or gynecological disorders, inflammatory disease or autoimmunity, allergic or atopic diseases, malignancies, transplant complications, and others. More specifically, abnormal levels of TNF α appear to play a pathogenic role in AIDS (Folks et al., 1989, Proc. Natl. Acad. Sci. USA 86:2365); graft-versus-host disease (Piguet et al., 1987, J. Exp. Med. 166:1280); cerebral malaria (Grau et al., 1987, Science 237:1210); and rheumatoid arthritis (Brennan et al., 1989, Lancet ii:244; Elliot et al., 1994, Lancet 344:1105), among others.

Human TNF α is initially synthesized as a membrane-bound precursor of approximately 26 kDa. A soluble mature 17 kDa peptide is released from the precursor after enzymatic cleavage by TNF α -con of the bond between TNF α precursor residues Ala₇₆ and Val₇₇. The TNF α precursor lacks a standard signal sequence. However, secretory vesicle transport events may be coupled to processing since mutation of the Ala₇₆-Val₇₇ cleavage site prevents secretion (Perez et al., 1990, Cell 63:251-258).

Mohler et al., 1994, Nature 370:218-220, partially purified TNF α -con from a human monocytic cell line, THP-1, and showed it to be a Zn²⁺-dependent metalloproteinase.

Gearing et al., 1994, Nature 370:555-557, demonstrated that matrix metalloproteinases, such as collagenase, could also process TNF α precursor at the correct cleavage sequence. In addition, inhibitors of proteases, such as serine proteases, serine/cysteine proteases, cysteine proteases and aspartyl

proteases, were shown to be ineffective at inhibiting TNF α -con activity. McGeehan et al., 1994, Nature, 370:558-561, showed that compound GI 129471, a hydroxamic acid-related inhibitor, which targets the highly conserved Zn²⁺ binding motif, HEXGH, of metalloproteinases, is a specific inhibitor of TNF α -con activity. Finally, Becherer et al., 1995, J. Cell. Biochem. Supp. 0(19B):253, reported TNF α -con enzymatic activity in a microsomal preparation that was inhibited by GI 129471, and the isolation of a putative TNF α -con by photoaffinity chromatography utilizing a photoreactive crosslinking derivative of GI 129471.

Various approaches have been proposed to control TNF α levels in patient sera, including the use of monoclonal or chimeric antibodies to bind and neutralize excess TNF α . See, for example, U.S. Pat. No. 5,231,024; U.S. Pat. No. 5,360,716; U.S. Pat. No. 5,436,154; U.S. Pat. No. 5,447,851; WO 92/11383; EP No. 0 366 043; EP No. 0 492 448 A1; and EP No. 0 585 705 A1. An alternative approach to bind up excess TNF α is to administer modified versions of the TNF α receptor. See WO 92/07076; WO 94/06476; and EP No. 0 418 014 A1. One difficulty with these approaches involves the potential for eliciting an immune reaction against the administered antibody or peptide molecule. Alternatively, U.S. Pat. No. 5,344,915 discloses the isolation of soluble TNF α binding proteins from human urine.

A further approach is the expression of TNF α anti-sense mRNA in cells that otherwise produce TNF α to bind to and prevent the translation of TNF α precursor mRNA. See No. EP 0 414 607 A2.

Yet another approach involves identifying and administering chemical compounds that specifically inhibit the synthesis or cellular secretion of TNF α . U.S. Patent No. 5,385,901 discloses the use of thalidomide and related compounds to specifically inhibit the production of TNF α . Mohler et al., 1994, above, specifically inhibited the release of TNF α using hydroxamic acid-related compounds, and were thereby able to increase the rate of survival of mice

injected with an otherwise lethal dose of LPS plus D-galactosamine. Gearing et al., 1994, above, and McGeehan et al., 1994, above, both identified additional hydroxamic acid-related inhibitors that specifically blocked TNF α secretion.
5 See also WO'95/06031.

Screening for compounds that inhibit TNF α -con would be facilitated by a readily available and abundant source of purified TNF α -con. Such inhibitors would be useful to reduce or otherwise modulate TNF α levels in a mammalian subject,
10 thereby treating diseases or conditions resulting from abnormal levels of TNF α . In addition, the availability of large quantities of TNF α -con would facilitate the development and identification of derivatives, analogs and peptides of TNF α -con that could serve to modulate TNF α levels in a
15 mammalian subject in need of such treatment. Accordingly, it would be useful to provide compositions and methods with which to produce large quantities of isolated TNF α -con.

3. SUMMARY OF THE INVENTION

20 The present invention is directed to TNF α , and more specifically to TNF α -con having biological activity to convert TNF α precursor to mature TNF α . The present invention provides cDNA sequences encoding enzymatically active mammalian TNF α -con, and more specifically a cDNA sequence
25 encoding human TNF α -con. The present invention also provides DNA sequences encoding derivatives, analogs or peptides of mammalian TNF α -con polypeptides that are substantially similar to mammalian TNF α -con and that exhibit biological activity.

30 The present invention further provides recombinant expression vectors comprising said DNA sequences, host cell lines comprising said DNA sequences or expression vectors, and recombinantly expressed, enzymatically active TNF α -con, or functional equivalents thereof.

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The present invention further provides compounds that inhibit the biological activity of TNF α -con, which may be useful for treating diseases or conditions related to abnormal levels of TNF α .

5 The present invention further provides novel modified inhibitors for use as ligands in the affinity purification of TNF α -con.

4. BRIEF DESCRIPTION OF THE DRAWINGS

10 FIG. 1. cDNA sequence (SEQ ID NO 1) encoding human TNF α -con and corresponding deduced amino acid sequence (SEQ ID NO 2). Asterisks show the termination codon. Amino acid residues 405-409 represent the conserved sequence of the zinc-binding motif of metalloproteinases.

15 FIG. 2A. Enzyme activity, as determined by cleavage of a synthetic substrate, of fractions collected from a glycerol gradient after affinity purification of porcine TNF α -con. FIG. 2B. SDS-PAGE analysis of fractions collected from the glycerol gradient corresponding to those
20 tested in FIG. 2A.

 FIG. 3. Reducing SDS-PAGE analysis of porcine TNF α -con before and after deglycosylation.

 FIG. 4A. Enzyme activity, as determined by cleavage of a synthetic substrate, of fractions collected
25 from a glycerol gradient after affinity purification of human TNF α -con. FIG. 4B. SDS-PAGE analysis of fractions collected from the glycerol gradient corresponding to those tested in FIG. 4A.

 FIG. 5. A contiguous mapping of clones pSC-1, pSC-
30 2, pSC-3 and pSC-5, obtained by screening a porcine spleen cDNA library with probes specific to the porcine TNF α -con coding sequence. Sequence comparison showed that the four clones overlap. The map represents the entire length of 2,414 bases from the four clones.

35 FIG. 6. cDNA sequence (SEQ ID NO 9) encoding a portion of the major open reading frame of porcine TNF α -con and corresponding deduced partial amino acid sequence (SEQ ID

NO 10). Asterisks show the termination codon. Amino acids 8-12 represent the conserved sequence of the zinc-binding motif of metalloproteinases.

FIG. 7. Contiguous map of positive clones 5 isolated from cDNA libraries from human leukocytes (hc7, hc9, hc11), and from human monocytes (3'#1, 3'#4, 3'#5, 5'#4, 5'#7). hc7 contains two internal deletions at base pairs 525-613 and 2156-2294.

FIG. 8. Structure of a biotinylated hydroxamic 10 acid-related compound useful in the affinity purification of TNF α -con.

FIG. 9. Stepwise (A-I) synthesis of a biotinylated hydroxamic acid-related compound (I).

FIG. 10. Stepwise synthesis of reagent C used 15 in the synthesis procedure shown in FIG. 9.

FIG. 11. Sequence of RACE14 clone (SEQ ID NO 39).

5. DETAILED DESCRIPTION OF THE INVENTION

20 5.1. DNA SEQUENCES ENCODING TNF α -CON

The nucleic acid sequences which can be used in accordance with the invention include but are not limited to any nucleic acid sequence that encodes a TNF α -con or a functional equivalent thereof, including: (a) any 25 nucleotide sequence that is complementary to a nucleotide sequence that hybridizes to a mammalian TNF α -con coding sequence under highly stringent conditions, e.g., washing in 0.1 x SSC/0.1 % SDS at 68°C (Ausubel et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, 30 Greene Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at page 2.10.3.), and encodes a product that exhibits a biological activity characteristic of a mammalian TNF α -con; (b) any nucleotide sequence that hybridizes to a nucleotide 35 sequence that is complementary to a mammalian TNF α -con coding sequence under less stringent conditions, such as moderately stringent conditions, e.g., washing in 0.2 x

SSC/0.1 % SDS at 42°C (Ausubel et al., 1989, above), and encodes a product that exhibits a biological activity characteristic of a mammalian TNF α -con; and (c) any nucleotide sequence that encodes a product that exhibits
5 a biological activity characteristic of a mammalian TNF α -con. As used herein, the term "TNF α -con" refers to a naturally occurring mammalian TNF α -con polypeptide and all functional equivalents thereof, unless otherwise noted.

10 For purposes of the present invention, "functional equivalents" of TNF α -con encompass all derivatives, analogs and peptides, as those terms are used in the art, of a mammalian TNF α -con that are substantially similar to the mammalian TNF α -con
15 polypeptide and exhibit a biological activity of a mammalian TNF α -con.

As used herein, the term "substantially similar" means that a particular amino acid sequence varies from the amino acid sequence of a naturally
20 occurring mammalian TNF α -con sequence by one or more amino acid substitutions, deletions, additions, or truncations, or by the addition of one or more chemical functional groups, or by some combination thereof. A peptide or polypeptide is considered to be substantially
25 similar to a mammalian TNF α -con if its amino acid sequence is at least about 50% homologous to the corresponding amino acid sequence of the mammalian TNF α -con, and more preferably is at least about 80% homologous to the corresponding amino acid sequence of the mammalian
30 TNF α -con. A polypeptide that is substantially similar to a mammalian TNF α -con will also preferably exhibit at least one type of biological activity characteristic of a mammalian TNF α -con.

For the purposes of this invention, "biological
35 activity" as applied to TNF α -con encompasses: (1) the ability to proteolytically cleave a mammalian TNF α precursor at a cleavage site corresponding to the peptide

bond between residues Ala₇₆ and Val₇₇ of human TNF α precursor; or (2) the ability to cleave an equivalent peptide bond in a synthetic substrate; or (3) the ability to detectably bind to TNF α precursor polypeptide, to
5 TNF α , or to a synthetic substrate comprising an equivalent peptide bond which is cleavable by a mammalian TNF α -con.

Any mammalian tissue or cell can serve as a source of a nucleotide sequence encoding TNF α -con for use
10 in molecular cloning. Since TNF α -con activity is required for the processing of TNF α precursor to mature TNF α , it can reasonably be inferred that any tissues or cells that produce mature TNF α will serve as a source for TNF α -con mRNA and TNF α -con polypeptide. For example, it
15 is known that TNF α is produced in a wide range of mammalian tissues and cells in response to various types of stimulation, including exposure to LPS. Such mammalian tissues include but are not limited to spleen and thymus. Specific mammalian cell types include but
20 are not limited to macrophages, monocytes, T-lymphocytes, β -lymphocytes, mast cells, polymorphonuclear leukocytes, keratinocytes, astrocytes, microglial cells, smooth muscle cells, intestinal paneth cells, and tumor cells including fibrosarcomas, epithelial tumor lines,
25 myelomas, T-cell leukemias, and the myeloid progenitors of acute and chronic myeloid leukemias, among others (Vassalli, 1992, above; Beutler and Cerami, 1989, above). For example, TNF α -con mRNA and polypeptides can be purified from LPS-stimulated cells of murine macrophage
30 cell line RAW 264.7, which can be obtained from the American Type Culture Collection (Rockville, Md.) (Accession No. TIB 71) (Jue et al., 1990, Biochemistry 29:8371-8377).

Likewise, any human cell can serve as a source
35 of a nucleotide sequence encoding TNF α -con for molecular cloning. For example, TNF α -con mRNA and polypeptide can be obtained from human monocytes which can be purified

from human blood, as for example by density centrifugation (Kriegler et al., 1988, Cell 53:45-53). An established human monocytic cell line that can be used as a source of TNF α -con mRNA and polypeptide is THP-1, 5 which can be obtained from the ATCC (Accession No. TIB 202).

The production of TNF α in any of the above cell lines or tissues can be determined either immunologically using anti-TNF α antibodies, which may be 10 produced by standard methods, or by utilizing any of several bioassays known in the art including, for example, an *in vitro* cytotoxicity assay using L-929 murine fibroblast cells, as described by Matthews and Neale, 1987, in: Clemens et al. (eds), Lymphokines and 15 Interferons: A Practical Approach, IRL, Oxford, pp. 221-225, which is incorporated herein by reference.

A nucleotide sequence encoding TNF α -con may be isolated from any of the above described tissues or cells by known methods, including but not limited to reverse 20 transcriptase-polymerase chain reaction (RT-PCR) from total mRNA to produce cDNA, or by screening cloned genomic DNA (e.g., a DNA library) with probes unique to the TNF α -con gene sequence. For example, a labelled probe derived from a TNF α -con cDNA may be used to isolate 25 a TNF α -con related gene by screening a genomic library. For example, using techniques known in the art, total mRNA can be isolated from any of the aforementioned tissues or cell types and reverse transcribed to produce cDNA, which is then screened, for example, with a 30 labelled probe. Such a probe can be designed based, for example, on a partial amino acid sequence of a TNF α -con polypeptide from the same or a different mammalian species, taking into account the genetic code and its known degeneracy.

35 TNF α -con can be isolated from any of the above-listed tissues or cells such as, for example, from mammalian spleen tissue according to procedures described

in Section 6.1 below. Briefly, mammalian spleen tissue is ground up in an appropriate buffer containing protease inhibitors at 4°C to obtain, through a series of steps, a membrane preparation which is then passed through a 5 concanavalin A (conA) column, which binds TNF α -con. Further purification of the eluted enzyme typically requires one or more affinity chromatography steps such as, for example, by contacting a partially purified TNF α -con preparation with a modified inhibitor of TNF α -con 10 under conditions that allow the binding of TNF α -con to the inhibitor, and isolating the TNF α -con-inhibitor conjugate.

A novel modified inhibitor is an hydroxamic acid-related inhibitor having the formula shown in FIG. 15 8, where R comprises a moiety that can be used for binding to a further compound so as to isolate any TNF α -con that binds to the inhibitor. For example, the moiety may be biotin. R may further comprise a spacer arm between the inhibitor and the moiety. The spacer arm can 20 be any chain length and may incorporate a disulfide bond between the hydroxamic acid inhibitor and the biotin moiety. For example, but not by way of limitation, R can be any of the following: $-(CH_2)_n$ -Biotin, where $n = 0-10$; $-(CH_2)_n$ -Imino Biotin, where $n = 0-10$; $-(CH_2)_n$ -S-S- $(CH_2)_n$ - 25 Biotin, where $n = 0-10$; or $-(CH_2)_n$ -S-S- $(CH_2)_n$ -Imino Biotin, where $n = 0-10$. Biotinylation of the inhibitor can be carried out by known methods. Preparation of the specific biotinylated inhibitor shown in FIG. 8 is described below (Section 7). Additionally, the present 30 invention encompasses the use of derivatives of biotin that may improve solubility or increase affinity for streptavidin.

Alternatively, TNF α -con can be isolated by photoaffinity chromatography using known methods and 35 utilizing, for example, a photoreactive crosslinking derivative of an inhibitor of TNF α -con, such as GI 129471, bound to a solid phase matrix.

TNF α -con isolated from porcine spleen has an apparent mass of about 85 kDa, which drops to about 62 kDa after deglycosylation.

Once the polypeptide is isolated, a complete or 5 partial amino acid sequence of the polypeptide may be obtained, e.g., by the Edman degradation procedure (see Creighton, 1983, Proteins, Structures And Molecular Principles, W.H. Freeman & Co., N.Y., pp. 34-49).

Degenerate oligonucleotide primers may then be designed 10 based on the complete or partial amino acid sequence, and used in a PCR to amplify a portion of the TNF α -con coding sequence from either a genomic or cDNA library. The amplified portion may then be used as a probe to obtain the full nucleotide coding sequence for TNF α -con.

15 For example, the following degenerate oligonucleotide PCR primers, designed as based on a 41 amino acid fragment of porcine TNF α -con, are useful to amplify a portion of a mammalian TNF α -con coding sequence: primer conv-1, having sequence 5'-GTI CA(A/G) 20 GA(T/C) GT(A/G) AT(T/C/A) GA-3' (SEQ ID NO 3); primer conv-2, having sequence 5'-GTI CA(A/G) GA(T/C) GT(T/C) AT(T/C/A) GA-3' (SEQ ID NO 4); and primer conv-3, having sequence 5'-CC IAC (A/G/T)AT (A/G)TT (A/G)TC (T/C)GC-3' (SEQ ID NO 5). For example, either primer conv-1 (SEQ ID 25 NO 3) or primer conv-2 (SEQ ID NO 4) can be used in combination with primer conv-3 (SEQ ID NO 5) to amplify by RT-PCR an 89 bp fragment (SEQ ID NO 8) from porcine spleen poly(A+) RNA encoding TNF α -con.

PCR amplification may be carried out by known 30 methods. See, for example, the techniques described in Innis et al. (eds), 1995, PCR Strategies, Academic Press, Inc., San Diego; and Erlich (ed) 1992, PCR Technology, Oxford University Press, New York, which are incorporated herein by reference. A PCR mixture comprising any 35 suitable primers, the nucleotide sequence to be amplified, and appropriate enzymes and buffers, are processed according to standard PCR protocols to amplify

the DNA sequence. Amplification may be carried out, for example, on a cDNA library that has been prepared by reverse transcription of porcine spleen poly(A+) mRNA using commercially available reagents such as, for example, a cDNA cycle kit from Invitrogen. The sequence of any amplified product may be determined to confirm that it corresponds to the complete or partial amino acid sequence of a TNF α -con. Such amplified sequences may then be used to screen for TNF α -con coding sequences in any mammalian cDNA or genomic library, including human. Once obtained, the full TNF α -con sequence can be characterized and used in molecular cloning, as described below.

In the molecular cloning of a TNF α -con DNA sequence, DNA fragments are typically generated, some of which will encode the desired TNF α -con sequence. To produce these fragments, the DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may fragment DNA in the presence of manganese, or physically shear the DNA, as for example by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to agarose and polyacrylamide gel electrophoresis (PAGE) and column chromatography.

Once DNA fragments are generated, identification of one or more specific DNA fragments containing the TNF α -con DNA sequence may be accomplished in a number of ways. For example, if an amount of a TNF α -con gene or its specific RNA, or a portion thereof, is detectably labeled, the generated DNA fragments may be screened by hybridization to the labeled probe.

Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence may be used in the practice of the invention for the cloning and expression of a TNF α -con protein. Such DNA sequences include those capable of hybridizing to a nucleotide

sequence that is complementary to the TNF α -con coding sequence under highly or moderately stringent conditions, as defined above. Stringency conditions may be adjusted in a number of ways. For example, when performing PCR, 5 the temperature at which annealing of primers to template takes place and/or the concentration of MgCl₂ in the reaction buffer may be adjusted. When using radioactively labeled DNA fragments or oligonucleotides in hybridization reactions, stringency may be adjusted by 10 changes in the ionic strength of the wash solutions and/or by careful control of the temperature at which the washes are carried out.

Altered nucleotide sequences which may be used in accordance with the invention include those comprising 15 deletions, additions or substitutions of different nucleotides resulting in a sequence that encodes the same or a functionally equivalent gene product. Alterations in the nucleotide sequence may result in changes, i.e., deletions, additions, substitutions or truncations, in 20 the amino acid sequence that may or may not be silent, but which produce a product that exhibits a biological activity characteristic of TNF α -con. Such nucleotide changes may be made taking into account similarities in the polarity, charge, solubility, hydrophobicity, 25 hydrophilicity and/or the amphipathic nature of the amino acid residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine. Amino acids with uncharged polar head groups 30 or nonpolar head groups having similar hydrophilicity values include the following: leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine; phenylalanine, tyrosine.

Once a DNA sequence encoding TNF α -con is 35 isolated, it can be amplified by any methods known in the art, including by chemical synthesis, PCR amplification, or cloning in a host cell. See, for example, the

techniques described in Maniatis et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates & Wiley Interscience, N.Y.; and Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which are incorporated herein by reference.

10 Utilizing PCR procedures and other techniques, a cDNA encoding human TNF α -con has now been cloned and sequenced (SEQ ID NO 1) (FIG. 1) that encodes the amino acid sequence (SEQ ID NO 2) also shown therein. In a specific embodiment of the invention (see Section 6,
15 below), the cDNA sequence for human TNF α -con was obtained by first purifying porcine TNF α -con, determining a partial amino acid sequence thereof, synthesizing degenerate PCR primers based on the partial porcine amino acid sequence, amplifying a fragment of the porcine TNF α -
20 con DNA coding region, and using the fragment as a probe to screen human leukocyte and monocyte cDNA libraries so as to isolate a full length cDNA sequence encoding human TNF α -con.

Once isolated, the TNF α -con DNA sequence of the
25 invention can be analyzed by known methods, including but not limited to Southern hybridization, Northern hybridization, restriction endonuclease mapping, and DNA sequence analysis. Southern hybridization with a TNF α -con specific probe can allow the detection of the TNF α -
30 con gene, either natural or introduced, in various cell types. Northern hybridization analysis can be used to determine the expression of the TNF α -con gene in different cell types, or at various stages of development or induction, for example. The stringency of the
35 hybridization conditions for both Southern and Northern hybridization can be manipulated to ensure detection of

nucleic acids with the desired degree of relatedness to the specific TNF α -con probe used.

Restriction endonuclease mapping can be used to roughly determine the genetic structure of the TNF α -con gene, and the extent of homology between the TNF α -con gene and other genes. Restriction maps derived by restriction endonuclease cleavage can be confirmed by DNA sequence analysis.

DNA sequence analysis can be performed by any techniques known in the art, including but not limited to the method of Maxam and Gilbert (1980, Meth. Enzymol. 65:499-560), the Sanger dideoxy method (Sanger et al., 1977, Proc. Natl. Acad. Sci. USA 74:5463), or use of an automated DNA sequenator (e.g., Applied Biosystems, Foster City, Ca.).

5.2. VECTORS TO DIRECT EXPRESSION OF DNA SEQUENCES ENCODING TNF α -CON

Once a DNA sequence encoding TNF α -con is obtained, it may be transferred directly into a host cell, or first inserted into an appropriate expression vector which is then transferred to a host cell, for propagation and expression. Such a vector is preferably constructed so that the TNF α -con coding sequence is in operative association with one or more regulatory elements necessary for transcription and translation of the coding sequence and production of a biologically active molecule.

As used herein, the term "regulatory element" includes but is not limited to inducible and non-inducible promoters, enhancers, operators and other elements known in the art that serve to drive and/or regulate expression. Also, as used herein, a DNA coding sequence is in "operative association" with one or more regulatory elements where the regulatory elements effectively regulate and allow for the transcription of

the DNA coding sequence and/or the translation of its mRNA.

Methods known in the art can be used to construct expression vectors containing the TNF α -con DNA 5 sequence in operative association with appropriate regulatory elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. See, for example, the techniques described in Maniatis, et al., 1989, above; 10 Ausubel et al., 1989, above; and Sambrook et al., 1989, above.

A variety of host expression vector systems, preferably those which contain the necessary regulatory elements for directing the replication, transcription, 15 and translation of a TNF α -con coding sequence, may be utilized equally well by those skilled in the art to express the TNF α -con coding sequence. These host expression vector systems include but are not limited to microorganisms such as bacteria transformed with 20 recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the TNF α -con coding sequence; yeast transformed with recombinant yeast expression vectors containing the TNF α -con coding sequence; insect cell systems infected with recombinant 25 virus expression vectors, e.g., baculovirus, containing the TNF α -con coding sequence; or animal cell systems infected with recombinant virus expression vectors, e.g., adenovirus or vaccinia virus containing the TNF α -con sequence, and include cell lines engineered to contain, 30 for example, multiple copies of the TNF α -con coding sequence either stably amplified, e.g., CHO/dhfr, or unstably amplified in double-minute chromosomes, e.g., murine cell lines.

The regulatory elements of these vectors may 35 vary in their strength and specificities. Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements may be

used. For instance, when cloning in mammalian cell systems, promoters isolated from the genome of mammalian cells, e.g., mouse metallothionein promoter, or from viruses that grow in these cells, e.g., vaccinia virus 5 7.5K promoter or Moloney murine sarcoma virus long terminal repeat, may be used. Promoters obtained by recombinant DNA or synthetic techniques may also be used to provide for transcription of the inserted sequences.

Illustrative transcriptional regulatory regions 10 or promoters include for bacteria, the β -gal promoter, the T7 promoter, the TAC promoter, λ left and right promoters, trp and lac promoters, trp-lac fusion promoters, etc.; for yeast, glycolytic enzyme promoters, such as ADH-I and -II promoters, GPK promoter, PGI 15 promoter, TRP promoter, etc.; for mammalian cells, SV40 early and late promoters, adenovirus major late promoters, etc.

Specific initiation signals are also required for sufficient translation of inserted protein coding 20 sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire TNF α -con gene, including its own initiation codon and adjacent sequences, are inserted into the appropriate expression vector, no additional translational control 25 signals may be needed. However, in cases where only a portion of the coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame 30 of the TNF α -con coding sequences to ensure in-frame translation of the entire insert. These exogenous translational control signals and initiation codons can be obtained from a variety of sources, both natural and synthetic. In addition, the efficiency of expression may 35 be enhanced by the inclusion of transcription attenuation sequences, enhancer elements, etc. For example, in cases where an adenovirus is used as an expression vector, the

TNF α -con coding sequence may be ligated to an adenovirus transcription/ translation control complex, e.g., the late promoter and tripartite ladder sequence. This chimeric gene may then be inserted into the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome, e.g., region E3 or E4, will result in a recombinant virus that is viable and capable of expressing TNF α -con in infected hosts. Similarly, the vaccinia 7.5K promoter may be used.

An alternative expression system which could be used to express TNF α -con is an insect system such as, for example, where *Autographa californica* nuclear polyhydrosis virus (AcNPV) is used as a vector to express the foreign sequence. The virus grows in *Spodoptera frugiperda* cells. The TNF α -con coding sequence may be cloned into a non-essential region, e.g., the polyhedrin gene of the virus, and placed under the control of an AcNPV promoter such as, for example, the polyhedrin promoter. Successful insertion of the TNF α -con coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus, i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene. These recombinant viruses may then be used to infect *Spodoptera frugiperda* cells in which the inserted gene is to be expressed.

Alternatively, retroviral vectors prepared in amphotropic packaging cell lines permit high efficiency expression in numerous cells types. This method allows the assessment of cell-type specific processing, regulation, or function of the inserted protein coding sequence.

A host cell strain may be chosen which modulates the expression of the inserted sequence, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers, e.g.,

zinc and cadmium ions for metallothionein promoters. Expression of the genetically engineered TNF α -con may thus be controlled. This is important if the protein product of the cloned foreign gene is lethal to host
5 cells. Furthermore, modifications such as, for example, phosphorylation or glycosylation, and processing, such as cleavage of protein products may be important for the biological activity of the protein. Different host cells have characteristic and specific mechanisms for the post-
10 translational modification and processing of an expressed polypeptide. For example, modifications in the glycosylation pattern may be important for different functions of the protein. Thus, expression in a bacterial system will produce an unglycosylated "core"
15 protein product. Expression in yeast will produce a glycosylated product. Expression in a mammalian cell, e.g., in COS cells, can be used to ensure "native" glycosylation of the heterologous TNF α -con polypeptide. Such variations may or may not result in changes to one
20 or more biological properties of the polypeptide. For example, it is possible that a TNF α -con polypeptide expressed in a particular cell type will retain the ability to bind to, but lack the ability to cleave, TNF α precursor. Such variously processed TNF α -con
25 polypeptides fall within the scope of the present invention. Thus, the cell line or host system may be chosen to ensure the desired modification and processing of the expressed protein.

Fusion protein expression vectors may be used
30 to express a TNF α -con fusion protein. The purified TNF α -con fusion protein may be used to raise antisera against the TNF α -con protein, to study the biochemical properties of the TNF α -con protein, to engineer TNF α -con fusion proteins with different enzymatic activities, or to aid
35 in the identification or purification of the expressed protein. Possible fusion protein expression vectors include but are not limited to vectors that express β -

galactosidase and trpE fusions, maltose-binding protein fusions, glutathione-S-transferase fusions and polyhistidine fusions (carrier regions). Methods known in the art can be used to construct expression vectors coding for such TNF α -con fusion proteins. See, for example, the techniques described in Maniatis, et al., 1989, above; Ausubel et al., 1989, above; and Sambrook et al., 1989, above.

The TNF α -con fusion protein may comprise a region that may be used for purification. For example, amylose resin may be used for purification of maltose binding protein fusions, or glutathione-agarose beads may be used for purification of glutathione-S-transferase fusion proteins, or divalent nickel resin can be used for the purification of polyhistidine fusions.

Alternatively, antibodies against a carrier protein or peptide may be used for affinity chromatography purification of the fusion protein. For example, a nucleotide sequence coding for the target epitope of a monoclonal antibody may be engineered into the expression vector in operative association with the regulatory elements and situated so that the expressed epitope is fused to the TNF α -con polypeptide. For example, but not by way of limitation, a nucleotide sequence coding for the FLAG™ epitope tag (International Biotechnologies Inc., IBI), which is a hydrophilic marker peptide, can be inserted by standard techniques into the expression vector at a point corresponding, for example, to the carboxyl terminus of the TNF α -con polypeptide. The expressed TNF α -con-FLAG™ epitope fusion product may then be detected and affinity-purified using commercially available anti-FLAG™ antibodies (IBI).

The expression vector may also be engineered to contain polylinker sequences that encode specific protease cleavage sites so that any cloned protein may be released from the carrier region by treatment with a specific protease. For example, DNA sequences encoding

the thrombin or factor Xa cleavage sites may be included in the fusion protein vectors.

A signal sequence upstream from and in reading frame with the polypeptide coding sequence may be engineered into the expression vector by known methods to direct the trafficking and secretion of the expressed protein. Non-limiting examples of signal sequences include those from α -factor, immunoglobulins, outer membrane proteins, penicillinase and T-cell receptors, among others.

To aid in the selection of transformed or transfected host cells, the expression vector may be engineered to further comprise a coding sequence for a reporter gene product or other selectable marker. Such a coding sequence should preferably be in operative association with the regulatory element coding sequences as described above. Reporter genes which may be useful in the invention are well-known in the art and include those encoding chloramphenicol acetyltransferase (CAT), firefly luciferase, and human growth hormone, among others. Coding sequences that encode selectable markers useful in the invention are also well-known in the art and include those that encode gene products conferring resistance to antibiotics or anti-metabolites, or that supply an auxotrophic requirement. Examples of such sequences include those that encode thymidine kinase activity or resistance to methotrexate, among others.

The expression vector can be additionally engineered according to known methods to enhance or optimize polypeptide expression, such as by mutating DNA regulatory elements to increase promoter strength or to alter the polypeptide coding sequence itself. Other modifications may include deleting intron sequences or excess non-coding sequences from the 5' and/or 3' ends of the polypeptide coding sequence in order to minimize sequence- or distance-associated negative effects on

expression of the polypeptide, e.g., by minimizing or eliminating message destabilizing sequences.

In addition, vectors can be engineered to contain a unique protease cleavage sequence downstream of the 5' end. For example, a protease sequence such as the thrombin cleavage sequence could be placed such that cleavage will produce an active, truncated TNF α -con.

5.3. TRANSFORMATION/TRANSFECTION OF HOST CELLS AND TNF α -CON EXPRESSION

10

The recombinant expression vector comprising a DNA sequence encoding TNF α -con is preferably transformed or transfected into one or more cells of a substantially homogeneous culture of a suitable host microorganism or insect or mammalian cell line. The expression vector may be introduced into the host cell in accordance with known techniques, including but not limited to transformation using calcium phosphate-precipitated DNA, microinjection of DNA, electroporation, transfection by contacting the cells with a virus, liposome-mediated transfection, DEAE-dextran transfection, transduction, conjugation, microprojectile bombardment, etc.

Once the expression vector is introduced into the host cell, the integration and maintenance of the polypeptide coding sequence into the host cell genome, or episomally, can be confirmed by standard techniques, e.g., by Southern hybridization analysis, PCR analysis, including reverse transcriptase-PCR (RT-PCR), or by immunological assays for the expected protein products.

30

Host cells containing the recombinant TNF α -con coding sequence and that express biologically active product may be identified by at least four general approaches: (i) DNA-DNA, DNA-RNA or RNA-antisense RNA hybridization; (ii) detecting the presence or absence of "marker" gene functions; (iii) assessing the level of transcription as measured by the expression of TNF α -con mRNA transcripts in the host cell; and (iv) detecting the

presence of mature gene product as measured, for example, by immunoassay or by the presence of biological activity.

In the first approach, the presence of the TNF α -con DNA sequence can be detected by nucleotide hybridization using labeled probes that are homologous to the TNF α -con coding sequence.

In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions, e.g., thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc. For example, if the TNF α -con coding sequence is inserted within a marker gene sequence of the vector, recombinants containing the TNF α -con coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the TNF α -con sequence under the control of the same or different promoter used to control the expression of the TNF α -con coding sequence.

Expression of the marker in response to induction or selection indicates expression of the TNF α -con coding sequence. For example, but not by way of limitation, expression of the FLAG™ epitope, the coding sequence of which can be placed in tandem with the TNF α -con sequence as described above, is detectable in cell extracts using anti-FLAG M2 monoclonal antibodies (IBI) in conjunction, for example, with the Western Exposure™ chemi-luminescent detection system (Clontech).

In the third approach, transcriptional activity of the TNF α -con coding region can be assessed by hybridization assays. For example, total cellular mRNA can be isolated and analyzed by Northern blot using a probe that is homologous to the TNF α -con coding sequence or to particular portions thereof.

In the fourth approach, the expression of the mature protein product can be assessed immunologically,

as for example by Western blots, radio-immunoprecipitation, enzyme-linked immunoassays and the like. Alternatively, protein expression can be confirmed and further characterized by histochemical localization and further characterized by histochemical localization using known methods. See, for example, Bullock and Petrusz (eds), 1982, Techniques in Immunocytochemistry, Vol I, Academic Press, Inc., London, which is incorporated herein by reference. For example, but not by way of limitation, cells or tissues transformed with an expression vector of the invention can be sectioned, and the sections probed with either polyclonal or monoclonal primary antibodies raised against the polypeptide. Bound primary antibodies may then be detected by standard techniques, e.g., using the biotinylated protein A-alkaline phosphatase-conjugated streptavidin technique, or a secondary antibody bearing a detectable label that binds to the primary antibody.

An important test of the success of the expression system involves the detection of TNF α -con exhibiting a biological activity. One of the biological activities associated with TNF α -con protein is its ability to enzymatically convert TNF α precursor to mature TNF α . Another is the ability of TNF α -con to cleave a synthetic substrate. Accordingly, non-limiting methods for detecting the presence of biologically active TNF α -con include detecting the conversion of TNF α precursor to mature TNF α or the cleavage of a synthetic substrate. For example, but not by way of limitation, TNF α -con biological activity can be followed by chromatographically detecting cleavage of a synthetic substrate such as, for example, DNP-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-NH₂ (SEQ ID NO 14) (DNP = dinitrophenylalanine), using high performance liquid chromatography (HPLC). This synthetic substrate spans the cleavage site of human TNF α precursor. Enzyme activity may be assayed by incubating an enzyme preparation with synthetic substrate (50 μ M) in a buffer

such as 0.25 M sucrose and 10 mM HEPES, pH 7.5; or in 50 mM HEPES, pH 7.5, 150 mM KCl, 5 μ M ZnSO₄, and 2 mM CaCl₂ containing DNP-Ser (20 μ M) (used as a standard to correct for injection errors), in a final volume of 100 μ l. The
5 buffer may also contain the following protease inhibitors: leupeptin (10 μ M), pepstatin (1 μ M), phosphoramidon (10 μ M), AEBSF (1 mM) and E-64 (10 μ M) (Sigma or Calbiochem). The assay is preferably carried out at 37°C for 15 min to 3 hr. The reaction may then be
10 quenched by addition of an equal volume of 1% heptafluorobutyric acid (HFBA). To distinguish between general proteolytic activity and activity due specifically to TNF α -con, duplicate samples are run containing a hydroxamic acid-related inhibitor, such as
15 GI 129471, which is a specific inhibitor of TNF α -con (McGeehan et al., 1994, above). Analysis of proteolytic activity may be carried out by separating and detecting substrate and products, for example, by HPLC on a C-18 Vyadac column using a water/acetonitrile gradient from 22
20 to 35% acetonitrile, with both the water and acetonitrile containing 0.1% HFBA.

Alternatively, TNF α -con activity may be detected by tracking a fluorescent tag after cleavage of a synthetic substrate, such as Z-Ser-Pro-Leu-Ala-Gln-Ala-
25 Val-Arg-Ser-Lys(X)-Ser-Arg (SEQ ID NO 17), where Z is a fluorophore such as NBD (6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino) or rhodamine, and X, which is linked as a side group to Lys, is, for example, dimethyl coumarin (DMC).

30 Alternatively, TNF α -con activity may be detected using a radiolabelled precursor. For example, TNF α precursor polypeptide may be radiolabelled by incorporation of ³⁵S-cysteine using an *in vitro* transcription/translation kit (Promega). The
35 radiolabelled substrate is preferably incubated for 1-3 hr at 37°C with the TNF α -con preparation in a buffer containing 0.1-1.0% NP-40, 0.25M sucrose, 10 mM HEPES, pH

7.5, and protease inhibitors (10 μ M leupeptin, 10 μ M phosphoramidon, 1 mM AEBSF, 10 μ M E-64, 1 μ M pepstatin, 10 μ M diprotinin A, 10 μ M amstatin, 10 μ M bestatin and 10 μ M diprotinin B). The reaction may be quenched by adding
5 loading buffer containing 4% SDS, 200 mM dithiothreitol, 20% glycerol, and 0.2% bromophenol blue. Samples are boiled, loaded onto polyacrylamide gels to separate substrate from product, and visualized using a phosphorimager (Molecular Dynamics, model 425F).

10 Alternatively, TNF α -con activity may be determined indirectly using an *in vitro* bioassay that detects the presence of TNF α . Any one of several bioassays known in the art can be used, such as a cytotoxicity assay using L-929 murine fibroblast cells
15 (Matthews and Neale, 1987, above).

5.4. PURIFICATION AND CHARACTERIZATION OF EXPRESSED TNF α -CON

Once the structural gene has been stably
20 introduced into appropriate host cells, the host cells may be grown under conditions conducive to maximum production of a biologically active TNF α -con. Such conditions will typically include growing cells to high density. Where the expression vector comprises an
25 inducible promoter, induction conditions may be employed such as, for example, temperature change, exhaustion of nutrients, accumulation of excess metabolic by-products, or the like, as appropriate to induce expression.

Where the expressed protein is retained in the
30 host cells, the cells are harvested, lysed and the product isolated and purified from the lysate under extraction conditions known in the art to minimize protein degradation, such as, for example, at 4°C, or with protease inhibitors, or both. Where the expressed
35 protein is secreted, the exhausted nutrient medium may simply be collected and the product isolated therefrom.

The expressed protein may be purified using standard methods, including but not limited to any combination of the following methods: ammonium sulfate precipitation, size fractionation, ion exchange chromatography, e.g., on a DEAE-cellulose column, HPLC, density centrifugation, and affinity chromatography. TNF α -con may be affinity-purified by binding the polypeptide to: (a) a monoclonal antibody raised against the polypeptide; (b) a lectin, such as conA; (c) TNF α or its precursor; or (d) a TNF α -con inhibitor, for example, a hydroxamic acid-related inhibitor such as GI 129471 (see McGeehan et al., 1994, above), among others. For example, but not by way of limitation, TNF α -con can be affinity-purified using a biotinylated hydroxamic acid-related inhibitor prepared as described below (see Section 7). Briefly, a cell lysate, exhausted culture medium or partially purified enzyme preparation comprising TNF α -con may be contacted with a biotinylated TNF α -con inhibitor under conditions conducive to binding of TNF α -con to the biotinylated inhibitor to form a TNF α -con-inhibitor-biotin conjugate. The TNF α -con-inhibitor-biotin conjugate may then be isolated by contacting it with streptavidin bound to a solid phase matrix, such as ULTRALINK™ Immobilized Neutravidin Plus on 3M EMPHAZE™ Biosupport Medium ABI (Pierce), under conditions conducive to binding of the TNF α -con-inhibitor-biotin conjugate to the streptavidin. The enzyme may then be eluted, for example, by incubation of the support medium overnight in a low salt buffer such as 10 mM NaCl, with protease inhibitors, such as leupeptin (10 μ M), pepstatin (1 μ M), phosphoramidon (10 μ M), AEBSF (1 mM) and E-64 (10 μ M).

Increasing purity of the enzyme preparation can be monitored at each step of the purification procedure by known methods, such as by determining protein yield versus enzymatic activity after each successive

purification step. Purity can be assessed by electrophoretic or chromatographic techniques.

Once a TNF α -con polypeptide of sufficient purity is obtained, it may be characterized by standard methods, such as by determining its enzyme kinetics and substrate specificity. For example, the ability of a purified TNF α -con to cleave short peptides may be determined. These peptides will preferably span the cleavage sequence of TNF α precursor, but may be modified, for example, by the presence of amino acid substitutions, deletions or derivatizations, or by differences in substrate length (see Section 8, below).

The amino acid sequence of the TNF α -con protein can be deduced from the cDNA sequence or, alternatively, by direct sequencing of the protein, e.g., with an automated amino acid sequencer. The deduced amino acid sequence of human TNF α -con (SEQ ID NO 2) is depicted in FIG 1.

The TNF α -con protein sequence can be further characterized by a hydrophilicity analysis (Hopp and Woods, 1981, Proc. Natl. Acad. Sci. USA 78:3824), which can be used to identify hydrophobic and hydrophilic regions of the TNF α -con protein and, accordingly, the corresponding regions of the gene sequence which encode such regions.

Secondary structural analysis (Chou and Fasman, 1974, Biochem. 13:222) can be carried out to identify regions of TNF α -con that assume specific secondary structures. Biophysical methods such as X-ray crystallography (Engstrom, 1974, Biochem. Exp. Biol. 11:7-13), computer modeling (Fletterick and Zoller (eds) 1986, in: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), and nuclear magnetic resonance (NMR) may be used to map and study sites of interaction between TNF α -con and its substrate. Once these sites have been identified, the present invention provides means for promoting or

inhibiting this interaction, depending upon the desired biological outcome.. Based on the foregoing, given the physical information on the sites of interaction, compounds that modulate TNF α -con activity may be
5 elaborated by standard methods known in the field of rational drug design. In addition, methods known in the art, including enzyme digestion of glycolytic side chains, lectin binding, and NMR structural analysis, allow for analysis of the glycosylation pattern of the
10 expressed versus the naturally occurring enzyme.

The present invention includes other methods for identifying the specific site(s) on the TNF α -con polypeptide that interact with TNF α precursor. For example, site-directed mutagenesis of DNA encoding the
15 TNF α -con protein may be used to destroy, inhibit or otherwise alter the interaction between TNF α -con and TNF α precursor, thus producing variants such as TNF α -con antagonists.

In an embodiment of the invention, a series of
20 deletion mutants in the TNF α -con coding region may be constructed and analyzed to determine the minimum amino acid sequence requirements for binding to and proteolytically cleaving TNF α precursor or a synthetic substrate. Deletion mutants of the TNF α -con coding
25 sequence may be constructed using methods known in the art which include but are not limited to use of nucleases and/or restriction enzymes, site-directed mutagenesis techniques, etc. The mutated polypeptides may be assayed for their ability to bind to the TNF α precursor or to a
30 synthetic substrate, for example, by gel filtration assays.

In addition, derivatives, analogs and peptides related to TNF α -con can be chemically synthesized (Merrifield, 1985, Science 232:341-347). For example, a
35 peptide corresponding to a portion of TNF α -con that exhibits a desired biological activity can be made using a peptide synthesizer.

5.5. DERIVATIVES, ANALOGS AND PEPTIDES OF TNF α -CON

The production and use of derivatives, analogs and peptides related to TNF α -con are also within the scope of the invention and can be used, for example, in immunoassays, for immunizations, therapeutically, etc. Such molecules which retain, inhibit, or otherwise modulate a desired TNF α -con biological activity property can be used as agonists, antagonists, inhibitors or, more generally, as modulators of such an activity. The terms "agonist", "antagonist", "inhibitor" and "modulator" are used in a functional sense and are not intended to limit the invention to compounds having a particular mode of action. Derivatives, analogs and peptides related to TNF α -con can be tested for the desired biological activity by procedures such as those described above, including but not limited to detecting substrate binding and/or cleavage, or by use of one or more *in vitro* TNF α bioassays.

The derivatives, analogs and peptides of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur either at the gene or protein level, or both. At the gene level, for example, the cloned TNF α -con DNA sequence can be modified *in vitro* by any of numerous strategies known in the art. See Maniatis, et al., 1989, above; Ausubel et al., 1989, above; and Sambrook et al., 1989, above. Such modifications include but are not limited to endonuclease digestion, mutations to create or destroy translation, initiation, and/or termination sequences, or that create variations in the coding region, or any combination thereof. Any technique for mutagenesis known in the art can be used, including but not limited to *in vitro* site-directed mutagenesis (see, for example, Hutchinson et al., 1978, J. Biol. Chem. 253:6551).

As a result of alterations at the gene level, the expressed polypeptide may contain deletions,

additions or substitutions of amino acids which may or may not result in a silent change within the sequence to produce a biologically active variant.

Manipulation of the TNF α -con DNA sequence may also be made at the protein level. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to: substitution of one or more L-amino acids of the TNF α -con polypeptide with corresponding D-amino acids, amino acid analogs or amino acid mimics, e.g., so as to produce carbazates or tertiary centers; or specific chemical modification, as for example with cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH₄, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, etc.

An example of a peptide of TNF α -con would be a truncated version of TNF α -con which, for example, may be produced by removal of a transmembrane domain normally present in the native protein, so as to produce a soluble, secreted form of the enzyme, or that comprised only the catalytic domain of the enzyme. Another example would be a 5' truncation that would remove the putative cystein switch, thereby activating and/or enhancing convertase activity.

The TNF α -con polypeptide, or a peptide or analog thereof, may be derivatized by conjugation to the protein of other chemical groups, including but not limited to acetyl groups, glycosyl groups, lipids, and phosphates, among others. Such conjugation is preferably by covalent linkage at TNF α -con amino acid side chains and/or at the N-terminus or C-terminus of the polypeptide.

Water soluble polymers, especially polyethylene glycol, may be conjugated to TNF α -con to provide additional desirable properties while retaining, at least in part, a desired biological activity, such as TNF α antagonism. These additional desirable properties

include, for example, increased solubility in aqueous solutions, increased stability in storage, reduced immunogenicity, increased resistance to proteolytic degradation, and increased *in vivo* half-life. Water soluble polymers suitable for use with the peptides of the invention include polyethylene glycol homopolymers, polypropylene glycol homopolymers, copolymers of ethylene glycol with propylene glycol, wherein said homopolymers and copolymers are unsubstituted or substituted at one end with an alkyl group, polyoxyethylated polyols, polyvinyl alcohol, polysaccharides, polyvinyl ethyl ethers, and α, β -poly[(2-hydroxyethyl)-DL-aspartamide]. Polyethylene glycol is particularly preferred. Methods of making water-soluble polymer conjugates of proteins are described in, among other places, U.S. Pat. No. 4,179,337; U.S. Pat. No. 4,609,546; U.S. Pat. No. 4,261,973; U.S. Pat. No. 4,055,635; U.S. Pat. No. 3,960,830; U.S. Pat. No. 4,415,665; U.S. Pat. No. 4,412,989; U.S. Pat. No. 4,002,531; U.S. Pat. No. 4,414,147; U.S. Pat. No. 3,788,948; U.S. Pat. No. 4,732,863; U.S. Pat. No. 4,745,180; EP No. 152,847; EP No. 98,110; and JP No. 5,792,435.

Such derivatives, analogs and peptides may be used to compete with full length wild-type TNF α -con protein for binding to TNF α precursor, and in so doing serve to inhibit or antagonize TNF α -con activity. The inhibition of TNF α -con protein function by these antagonists may be useful to reduce TNF α levels in serum or tissues of a mammalian subject, thereby treating septic shock, cachexia or other diseases or conditions characterized by elevated or otherwise abnormal levels of TNF α . Alternatively, TNF α -con derivatives, analogs and peptides that are capable of binding to mature TNF α may be used to neutralize excess levels of TNF α in the serum or tissues of a mammalian subject in need of such treatment.

5.6. SCREENING FOR TNF α -CON INHIBITORS

Recombinantly expressed TNF α -con may be used to screen for molecules that reduce or otherwise modulate TNF α levels by inhibiting or otherwise modulating one or 5 more biological activities of TNF α -con. Such molecules may include small organic or inorganic compounds, antibodies, peptides, or other molecules that inhibit or otherwise modulate the ability of TNF α -con to: (1) bind to TNF α precursor or to a synthetic substrate; (2) 10 convert TNF α precursor to mature TNF α ; or (3) cleave a synthetic substrate. Of particular interest are hydroxamic acid-related compounds, several of which have been shown to inhibit TNF α -con activity. See Mohler et al., 1994, above; Gearing et al., 1994, above; McGeehan 15 et al., 1994, above; and WO 95/06031.

Synthetic compounds, natural products, and other potential sources of modulatory compounds can be screened in a number of ways. For example, the ability of a test molecule to inhibit the activity of TNF α -con 20 may be measured using standard biochemical methods such as gel filtration assays to detect an effect on binding, or assays that detect an effect on the cleavage of precursor peptides, or using *in vitro* bioassays that detect the presence of TNF α .

25 One non-limiting method by which a compound capable of binding to TNF α -con may be isolated and identified comprises: (a) conjugating TNF α -con, or a portion thereof, to a solid phase matrix; (b) contacting the TNF α -con-solid phase matrix conjugate with a material 30 comprising a test compound for an interval and under conditions sufficient to allow the compound to bind to the conjugated enzyme; (c) washing away unbound material from the solid phase matrix; (d) detecting the presence of compound bound to the conjugated TNF α -con; (e) eluting 35 the bound compound from the immobilized enzyme, and (f) collecting and thereby isolating the compound. Alternatively, the compound may first be eluted from the

immobilized enzyme and then detected and characterized. Once isolated, the compound can be tested for its ability to inhibit or otherwise modulate one or more biological activities of TNF α -con.

- 5 Random peptide libraries consisting of all possible combinations of amino acids may be used to identify peptides that are able to bind to TNF α -con. Identification of peptides that are able to bind to TNF α -con may be accomplished by screening such a peptide
10 library with recombinant TNF α -con proteins. Alternatively, any binding domains of TNF α -con may be separately expressed and used to screen peptide libraries.

- One non-limiting way to identify and isolate a
15 peptide that interacts and forms a complex with TNF α -con involves attaching a detectable label to TNF α -con protein to facilitate the identification of such a complex. Thus, TNF α -con may be conjugated to an enzyme such as alkaline phosphatase or horseradish peroxidase, or to a
20 fluorescent tag, such as fluorescein isothiocyanate (FITC), phycoerythrin (PE) or rhodamine, among others. Conjugation of a detectable label to TNF α -con may be performed using techniques that are routine in the art. Alternatively, TNF α -con expression vectors may be
25 engineered to express a TNF α -con fusion protein containing an epitope for which a commercially available antibody exists, such as the FLAG™ epitope as described above. The epitope-specific antibody may be tagged using methods well known in the art including, for example, by
30 labeling with enzymes, fluorescent dyes or colored or magnetic beads, or the epitope-specific antibody may be detected using a labelled secondary antibody.

- A DNA sequence encoding a peptide that interacts with TNF α -con to form a complex may be cloned
35 into an appropriate expression vector for overexpression in either bacteria or eukaryotic cells. The peptide may be purified from cell extracts by known methods.

Alternatively, the peptide may be synthesized by solid phase techniques followed by cleavage from resin and purification by HPLC. Once isolated, the peptide can be tested for its ability to inhibit or otherwise modulate the biological activity of TNF α -con.

5.7. ANTI-TNF α -CON ANTIBODY PRODUCTION

The production of polyclonal and monoclonal antibodies that bind to TNF α -con falls within the scope of the invention. Antibodies to TNF α -con may be useful, for example, as affinity reagents to purify native or recombinant TNF α -con, or to detect the presence of TNF α -con, for example, in histological sections, in cell or tissue extracts, in culture medium, or in enzyme preparations, or therapeutically to neutralize TNF α -con activity.

Either the entire TNF α -con polypeptide or a sub-sequence thereof may be used as immunogen against which antibodies can be raised. For example, the catalytic domain of the enzyme may be isolated and used as an immunogen against which antibodies can be raised.

For the production of antibodies, various host animals, including but not limited to rabbits, mice, rats, etc., may be immunized by injection with TNF α -con or a portion thereof. Immunizations are carried out according to known methods. Various adjuvants may be used to increase the immunological response, depending on the host species, including Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum, among others.

Monoclonal antibodies to TNF α -con may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines

in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein, (Nature, 1975, 256:495-497), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today, 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci. USA, 80:2026-2030) and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In addition, techniques developed for the production of "chimeric antibodies" by splicing genes, as for example from a mouse antibody molecule of appropriate antigen specificity, together with genes from a human antibody molecule of appropriate biological structure or activity, can be used (Morrison et al., 1984, Proc. Natl. Acad. Sci. USA 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454). Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778) can be adapted to produce TNF α -con-specific single chain antibodies.

Antibody fragments which contain specific binding sites for the TNF α -con protein may be generated by known techniques. For example, such fragments include but are not limited to: F(ab')₂ fragments which can be produced by pepsin digestion of the antibody molecule, and Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid identification of Fab fragments having the desired specificity to the TNF α -con protein.

Techniques for the production of monoclonal antibodies, antibody fragments, etc., are well-known in the art, and are additionally described in Harlow and Lane, 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, which is incorporated herein by reference.

5.8. ANTI-SENSE OLIGONUCLEOTIDES AND RIBOZYMES

Also within the scope of the invention are oligonucleotide sequences that include anti-sense oligonucleotides, phosphorothioates, and ribozymes that function to bind to, degrade and/or inhibit the translation of TNF α -con mRNA.

Anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the cDNA sequence encoding TNF α -con (FIG. 1) can be synthesized, for example, by conventional phosphodiester techniques.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage.

Engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of TNF α -con RNA sequences are also within the scope of the invention.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features such as secondary structure that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays, for example.

Both the anti-sense oligonucleotides and ribozymes of the invention may be prepared by known methods. These include techniques for chemical synthesis, such as for example by solid phase
5 phosphoamite chemical synthesis. Alternatively, anti-sense RNA molecules may be generated by *in-vitro* or *in-vivo* transcription of DNA sequences encoding the RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA
10 polymerase promoters such as the T7 or SP6 polymerase promoters.

Various modifications to the oligonucleotides of the invention may be introduced as a means of increasing intracellular stability and half-life.
15 Possible modifications include but are not limited to the addition of flanking sequences of ribo- or deoxyribo-nucleotides to the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2'-O-methyl rather than phosphodiesterase linkages within the oligonucleotide
20 backbone.

5.9. METHODS FOR THERAPEUTIC USES

Compounds that inhibit the conversion of TNF α precursor to mature TNF α or otherwise modulate the
25 effective levels of TNF α in the sera or tissues of a mammalian subject can be used to treat diseases or conditions related to elevated or otherwise abnormal levels of TNF α in the subject.

The term "treatment" as used herein with
30 reference to a disease or condition is used broadly and is not limited to a method of curing the disease or condition. The term "treatment" includes any method that serves to reduce one or more of the pathological effects or symptoms of a disease or condition, or to reduce the
35 rate of progression of one or more of such pathological effects or symptoms.

Diseases or conditions that may be treated by the methods of the invention are diseases characterized by one or more of the following criteria: elevated or otherwise abnormal levels of TNF α in serum or tissues of
5 a mammalian subject; the development of septic shock; or the development of cachexia. The terms "elevated" and "abnormal" as used herein are relative terms and are used to describe the levels of TNF α in a subject in need of treatment as compared to a normal subject of similar age,
10 gender, weight, etc.

The present invention provides methods for treating such diseases or conditions in a mammalian subject in need of such treatment, comprising administering to that subject an effective amount of a
15 compound that reduces or otherwise modulates the level or biological activity of TNF α in that subject. The compound can alter the absolute or effective amount of TNF α in the subject by any mechanism such as, for example, by inhibiting the ability of TNF α -con to convert
20 TNF α precursor to TNF α , or by inhibiting the cellular secretion of TNF α , or by binding to and thereby reducing the effective concentration of soluble TNF α in the serum or tissues of the subject.

Diseases or conditions that can be treated
25 according to the method of the present invention include systemic inflammatory response syndrome, reperfusion injury, cardiovascular disease, infectious disease, obstetrical or gynecological disorders, inflammatory disease or autoimmunity, allergic or atopic diseases,
30 malignancies, transplants, among others. More specifically, diseases or conditions which may be treated by the method of the present invention include but are not limited to septic shock, cachexia, AIDS, graft-versus-host disease, cerebral malaria, Crohn's disease,
35 diabetes, osteoporosis, restenosis, psoriasis and rheumatoid arthritis, macular degeneration, osteoarthritis, inflammatory bowel disease, and

autoimmune disease such as multiple sclerosis, among others. The method of the present invention may also be used to prevent or reduce the extent of infarction due, for example, to an ischemic event.

5 The present invention further contemplates the use of combination therapy, wherein one or more compounds that inhibit or otherwise modulate a biological activity of TNF α -con, as disclosed above, can be used in combination with one or more other reagents in the
10 treatment of a disease or condition in a mammalian subject. Such other reagents may include, for example, small organic or inorganic molecules or antibodies directed to TNF α -con or to another component or factor underlying a disease or condition in a mammalian subject,
15 in which combination therapy will serve to increase or otherwise improve the efficacy of treatment of the disease or condition. For example, one or more TNF α -con inhibitors may be used in conjunction with an antibody directed against a component of the inflammatory response
20 such as that involved in an autoimmune disease, such as, for example, rheumatoid arthritis, to increase the efficacy of treatment. For example, one or more TNF α -con inhibitors may be used in conjunction with an anti-CD4 antibody or with an anti-CD23 antibody to treat an
25 autoimmune disease. A humanized anti-CD4 antibody is disclosed in PCT GB 91/01578. Anti-CD23 antibodies are described in Dougall et al., 1994, Tibtech 12:372-379. Alternatively, one or more TNF α -con inhibitors may be used in conjunction with more conventional treatments,
30 such as with methotrexate or cyclosporin A, among others.

 The present invention further contemplates a complex comprising a TNF α -con and a therapeutic agent capable of modulating the activity of the enzyme for use in treating a disease or condition associated with TNF α .
35 Such a complex may further comprise the enzyme's substrate.

The present invention further provides pharmaceutical compositions or formulations for use in a method of treatment, comprising one or more compounds that reduce or otherwise modulate the effective level of 5 TNF α in sera or tissues of a mammalian subject and a pharmaceutically acceptable carrier. The invention further encompasses formulations for a combination therapeutic comprising one or more compounds that inhibit the biological activity of TNF α -con, one or more 10 compounds that inhibit some other component involved in a disease or condition in a mammalian subject to be treated, and a pharmaceutically acceptable carrier.

A variety of aqueous carriers may be used in the pharmaceutical formulation of the invention, such as 15 water, buffered water, 0.4% saline, 0.3% glycine, and the like. The pharmaceutical formulations may also comprise additional components that serve to extend the shelf-life of pharmaceutical formulations, including preservatives, protein stabilizers, and the like. The formulations are 20 preferably sterile and free of particulate matter (for injectable forms). These compositions may be sterilized by conventional, well-known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate 25 physiological conditions, such as pH adjusting and buffering agents, toxicity adjusting agents and the like, e.g., sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, etc. The formulations of the invention may be adapted for various 30 forms of administration, including orally, intramuscularly, subcutaneously, intravenously, intraocularly, and the like. Actual methods for preparing parenterally administrable compositions and adjustments necessary for administration to subjects will 35 be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 17th Ed., Mack Publishing

Company, Easton Pa (1985), which is incorporated herein by reference.

The present invention further provides formulations for the sustained release of one or more compounds that reduce or otherwise modulate the total or effective TNF α levels in a subject by inhibiting or otherwise modulating the biological activity of TNF α -con. Examples of such sustained release formulations include composites of biocompatible polymers, such as poly(lactic acid), poly(lactic-co-glycolic acid), methylcellulose, hyaluronic acid, collagen, and the like. The structure, selection and use of degradable polymers in drug delivery vehicles have been reviewed in several publications, including A. Domb et al., 1992, *Polymers for Advanced Technologies* 3:279-292. Additional guidance in selecting and using polymers in pharmaceutical formulations can be found in the text by M. Chasin and R. Langer (eds.), 1990, "Biodegradable Polymers as Drug Delivery Systems", in: Drugs and the Pharmaceutical Sciences, Vol 45, M. Dekker, New York.

Liposomes may also be used to provide for the sustained release of TNF α -con antagonists or other modulating compounds. Details concerning how to use and make liposomal formulations of drugs of interest can be found in, among other places, U.S. Pat. No 4,944,948; U.S. Pat. No. 5,008,050; U.S. Pat. No. 4,921,706; U.S. Pat. No. 4,927,637; U.S. Pat. No. 4,452,747; U.S. Pat. No. 4,016,100; U.S. Pat. No. 4,311,712; U.S. Pat. No. 4,370,349; U.S. Pat. No. 4,372,949; U.S. Pat. No. 4,529,561; U.S. Pat. No. 5,009,956; U.S. Pat. No. 4,725,442; U.S. Pat. No. 4,737,323; U.S. Pat. No. 4,920,016. Sustained release formulations are of particular interest when it is desirable to provide a high local concentration of a TNF α -con antagonist, for example, at the site of an infection, etc.

A purified TNF α -con inhibitor or other modulating compound may be combined with compatible,

nontoxic pharmaceutical excipients and administered to a mammalian subject, e.g., to treat a disease or condition characterized by an elevated or otherwise abnormal level of TNF α in the serum or tissues of the subject. The term

5 "mammalian subject" is intended to include humans and animals. In the case of administration to animals, it may be preferable to incorporate the drug into the animal's feed, possibly in a prepared combination of drug and nutritional material ready for use by the farmer.

10 The compound may be administered orally, rectally, transdermally, by pulmonary infiltration, insufflation or parenterally (including intravenously, subcutaneously and intramuscularly) to humans, in any suitable pharmaceutical dosage form.

15 An effective dosage and treatment protocol may be determined by conventional means, starting with a low dose in laboratory animals and then increasing the dosage while monitoring the effects, and systematically varying the dosage regimen as well. Numerous factors may be

20 taken into consideration by a clinician when determining an optimal dosage for a given subject. Primary among these is the level of TNF α in serum or tissue of the subject. Additional factors include the size of the subject, the age of the subject, the general condition of

25 the subject, the particular disease or condition being treated, the severity of the disease, the presence of other drugs in the subject, the *in vivo* activity of the antagonist or modulating compound and the like. The trial dosages would preferably be chosen after

30 consideration of the results of animal studies and the clinical literature with respect to administration of modulators of TNF α or TNF α -con. It will be appreciated by the person of ordinary skill in the art that information such as binding constants and K_i derived from

35 *in vitro* TNF α -con binding competition assays may also be used in calculating dosages.

A typical daily human dose of a TNF α -con antagonist or other modulating compound may be in an amount, for example, of from about 0.1 mg to about 200 mg per kilogram of body weight, more preferably from about 15 mg to about 100 mg per kilogram body weight, and most preferably about 5 mg to about 50 mg per kilogram body weight.

5.10. GENE THERAPY

10 Also within the scope of the present invention is the use of gene therapy to replace mutated TNF α -con with a wild type complement of the gene, or to transfer nucleotide sequences that are anti-sense to a portion of TNF α -con into a subject. Such examples of gene therapy
15 are useful to treat any disease or condition resulting from an elevated or otherwise abnormal level of TNF α or TNF α -con in the subject.

Methods for transferring the wild type TNF α -con gene into the targeted tissue may include reconstitution
20 of recombinant TNF α -con molecules into liposomes for delivery into target cells. Alternatively, recombinant viral vectors may be engineered to express wild type TNF α -con. Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus,
25 herpes virus or bovine papilloma virus, may be used to deliver wild type TNF α -con into the targeted cell population. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors containing TNF α -con coding sequence. See,
30 for example, the techniques described in Maniatis, et al., 1989, above; Ausubel et al., 1989, above; and Sambrook et al., 1989, above.

6. EXAMPLE: ISOLATION OF A
CDNA ENCODING HUMAN TNF α -CON

6.1. PURIFICATION OF PORCINE TNF α -CON

6.1.1. MATERIALS AND METHODS

5

Membrane Preparation

Porcine TNF α -con was isolated from pig spleen. All steps were conducted at 4°C. Approximately 10 to 20 fresh pig spleens, total 3-6 kilograms (kg), were cut
10 into small pieces and placed in a beaker containing cold grinding buffer (10 mM HEPES, pH 7.5, 0.25 M sucrose, 2 mM MgCl₂) with protease inhibitors (1 mM AEBSF, 1 μ M pepstatin, 10 μ M E-64, 10 μ M leupeptin, 10 μ M phosphoramidon, and 50 μ M DCI). For every volume of
15 spleen tissue, 3 volumes of grinding buffer were used. The pieces were homogenized in a 4L blender using 10 second bursts at low, medium, and then high speed. The ground tissue was passed through a fiberglass screen to filter the suspension. The material passing through the
20 filter was centrifuged in a GS-3 rotor for 10 min at 2000 x g. The supernatant was removed and CaCl₂ was added with stirring to a final concentration of 8 mM. Ten minutes after the CaCl₂ was dissolved, the solution was centrifuged in a GS-3 rotor for 20 min at 10,000 x g.
25 The pellet was resuspended in 1/6 the volume of the supernatant in buffer (10 mM HEPES, pH 7.5, 0.25 M sucrose) with protease inhibitors (1 mM AEBSF, 1 μ M pepstatin, 10 μ M E-64, 10 μ M leupeptin, 10 μ M phosphoramidon and 50 μ M DCI), and this preparation,
30 representing a membrane suspension, was quick frozen and stored at -70°C for future use.

Buffer B (0.05% NP-40, 10 mM HEPES, pH 7.5, 200 mM NaCl) with protease inhibitors (1 mM AEBSF, 1 μ M pepstatin, 10 μ M E-64, 10 μ M leupeptin, 10 μ M
35 phosphoramidon) was added to thawed membrane suspension so that the buffer:membrane volume ratio was 3:1. Three kg of pig spleen produced approximately 1L of membrane

suspension. After mixing the buffer and membrane suspension together, the membranes were pelleted by centrifugation in a TFA 20.250 rotor for 1 hr at 20,000 rpm. The pellet was combined with 6 liters of Buffer C 5 (Buffer B with 1% NP-40) with protease inhibitors (1 mM AEBSF, 1 μ M pepstatin, 10 μ M E-64, 10 μ M leupeptin, 10 μ M phosphoramidon) for every liter of initial membrane suspension. The solution was stirred for 30 min at 4°C followed by centrifugation in the TFA 20.250 rotor for 1 10 hr at 20,000 rpm.

Chromatography

The supernatant was loaded onto a 1 liter conA column (Pharmacia) equilibrated in the same buffer as the 15 supernatant at a flow rate of 20 ml per min. The column was washed with 5 volumes of Buffer C without protease inhibitors, followed by 5 volumes of Buffer D (same as Buffer C (without protease inhibitors) except that the NaCl concentration was 10 mM. Convertase activity, as 20 determined by HPLC monitoring of the cleavage of a synthetic DNP substrate, was eluted off with 10 column volumes of Buffer E (Buffer D containing 250 mM methyl mannopyranoside) with protease inhibitors (1 mM AEBSF, 1 μ M pepstatin, 10 μ M E-64, 10 μ M leupeptin).

25 The eluant was loaded directly onto a 500 ml Q fast flow column (Pharmacia). The column was washed with Buffer F (0.5% NP-40, and 10 mM HEPES, pH 7.5). Convertase activity was eluted off with Buffer F containing 200 mM or 500 mM NaCl and protease inhibitors 30 (1 mM AEBSF, 1 μ M pepstatin, 10 μ M E-64, 10 μ M leupeptin, 10 μ M phosphoramidon). The eluted protein was dialyzed against Buffer F without NaCl, but with protease inhibitors (1 mM AEBSF, 1 μ M pepstatin, 10 μ M E-64, 10 μ M leupeptin) overnight at 4°C. The material was then 35 loaded onto a 2 ml POROS™ HQ column (Perseptive Biosystems). Elutions were carried out with Buffer F containing 500 mM NaCl and protease inhibitors (1 mM

AEBSF, 1 μ M pepstatin, 10 μ M E-64, 10 μ M leupeptin, 10 μ M phosphoramidon). Fractions containing convertase activity were further purified by affinity chromatography.

5 Optionally, before the dialysis step above, the material from the Q-fast flow column was additionally purified with Cibacron Blue 3000 (Sigma). Briefly, the eluant was loaded onto a 300 ml column at a flow rate of 10 ml per min. The column was washed with 3 column
10 volumes of Buffer F containing 200 mM NaCl without protease inhibitors. The activity was then eluted from the column with Buffer F containing 1.5 M NaCl and protease inhibitors (1 mM AEBSF, 1 μ M pepstatin, 10 μ M E-64, 10 μ M leupeptin). The eluted protein was then
15 dialyzed as above.

Affinity Purification

For affinity purification, a biotinylated hydroxamic acid inhibitor, prepared as described below
20 (Section 7), was added to the dialyzed protein to a final concentration of 1 μ M. After 10-30 min incubation at 4°C, affinity beads (ULTRALINK™ Immobilized Neutravidin Plus on 3M EMPHAZE™ Biosupport Medium ABI (Pierce)) were added (0.4 ml of slurry per 1 ml of enzyme solution).
25 After incubating for 10 min with gentle rocking, the slurry was pelleted by centrifugation in a microfuge. The beads were washed three times with 1.5 ml of Buffer F containing 0.5 M NaCl. The enzyme was eluted off the beads by gentle rocking overnight in Buffer F containing
30 protease inhibitors (1 mM AEBSF, 1 μ M pepstatin, 10 μ M E-64, 10 μ M leupeptin) without NaCl at 4°C.

Glycerol Gradient

In order to demonstrate that the 85 kDa band
35 co-migrates with convertase activity, the TNF α -con was subjected to a final separation step consisting of sedimentation through a glycerol gradient under

centrifugal force as follows. The material eluted from the affinity resin was layered on top of an 8 to 27.8% glycerol gradient made as follows. The following glycerol dilutions in 10 mM HEPES, pH 7.55 plus 0.05% NP-40 were carefully layered in 12 ml polycarbonate ultracentrifugation tubes (Sorvall, cat. No. 03699): 27.8% (1.1 ml), 25.6% (1.1 ml), 23.4% (1 ml), 21.2% (1 ml), 19% (1 ml), 16.8% (1 ml), 14.6% (1 ml), 12.4% (1 ml), 10.2% (1 ml), 8% (0.8 ml). The tubes were stored at 4°C for 17 hrs to allow for the formation of a continuous gradient, and then 0.2 ml of eluted material was layered on top of each gradient. The gradients were centrifuged at 4°C for 48 hrs at 40,000 rpm in a Sorvall ultracentrifuge (model RC70).

After centrifugation, the gradients were fractionated by taking 0.35 ml aliquots from top to bottom. An aliquot (20 μ l) of each fraction was assayed for enzyme activity by detecting cleavage of a synthetic substrate by HPLC as described above. In addition, an aliquot (30 μ l) of each fraction was mixed with 10 μ l of Laemmli loading buffer (4x stock) and electrophoresed on a 10% polyacrylamide gel under denaturing conditions (Laemmli, 1970, Nature 227:680-685), followed by silver staining (Daichi silver staining kit), in order to correlate the activity of each fraction with proteins bands present in the gel.

Deglycosylation

Reagents for deglycosylation were obtained as a kit from New England Biolabs (Beverly, Mass.). An aliquot (25 μ l) of TNF α -con preparation obtained from pooled fractions 16-20 from the glycerol gradient step was incubated for 10 min at 100°C in the presence of 2.5 μ l 10x denaturing buffer. The following were then added: NP-40 (3.5 μ l); 10x G7 buffer (3.5 μ l); and pure PNGase F (0.5 μ l; 10 units). The reaction was run for 1 hr at 37°C and then stopped by the addition of 12 μ l of 4x

Laemmli loading solution, followed by electrophoresis on a 10% polyacrylamide gel which was then silver stained as described above.

5 Purification Of Human TNF α -Con

As a means of comparing the molecular mass of human TNF α -con with its porcine counterpart isolated as above, a purification scheme for human TNF α -con was developed based on the above-described procedure. All 10 steps were carried out at 4°C. Thus, a pellet containing 8.2×10^{10} MonoMac6 cells was resuspended to a final volume of 600 ml in grinding buffer. Cells were disrupted by cavitation by exposing them for 30 min to 1,000 psi of nitrogen gas, followed by rapid pressure 15 release. The lysate was centrifuged at 3,500 rpm for 10 min in a GS3 rotor (Sorvall). The supernatant was then centrifuged at 20,000 rpm for 45 min in a TFA20.250 rotor (Sorvall). The pellet was resuspended to 750 ml with buffer C with protease inhibitors (1 mM AEBSF, 1 μ M 20 pepstatin, 10 μ M E-64, 10 μ M leupeptin, 10 μ M phosphoramidon) containing 1.2% NP-40, and extracted by slow stirring for 30 min, followed by centrifugation for 45 min at 20,000 rpm in the same rotor to remove insoluble material. The supernatant was then loaded on a 25 100 ml conA-sepharose column at 10 ml/min. The column was washed with 200 ml buffer C without protease inhibitors, followed by 800 ml buffer D with protease inhibitors as above. The activity was then eluted with 850 ml buffer E with protease inhibitors as above. This 30 eluate was applied to a 2 ml POROS™ HQ column washed with 10 ml buffer F, eluted with protease inhibitors, as above, and containing 0.5 M NaCl, and 1 ml fractions were collected. Fractions 2 and 3 which contained over 90% of the TNF α -con activity were pooled. An aliquot (0.5 ml) 35 from this pool was incubated with 1 nmol biotinylated inhibitor as above for 15 min; then 0.1 ml slurry of affinity beads (ULTRALINK™ Immobilized Neutravidin Plus

on 3M EMPHAZE™ Biosupport Medium ABI (Pierce)) was added and incubated for 1 hr, after which the beads were washed three times with buffer F containing 0.5 M NaCl. The activity was eluted from the beads by overnight incubation with buffer F containing 0.1% NP-40 and protease inhibitors (1 mM AEBSF, 1 μ M pepstatin, 10 μ M E-64, 10 μ M leupeptin, 10 μ M phosphoramidon) without NaCl at 4°C. The eluted material was further purified on a glycerol gradient as described above. Enzyme activity and SDS-PAGE analysis of human TNF α -con were carried out as described above for porcine TNF α -con. Deglycosylation analysis of human TNF α -con was not carried out because of the limited amount of purified enzyme.

15

6.1.2. RESULTS

The purity of porcine TNF α -con prepared as above was assessed by SDS-PAGE under reducing conditions (FIG. 2B). Isolated porcine TNF α -con, prepared as above, has an apparent molecular weight of about 85 kDa, as shown by the correlation of enzyme activity with the 85 kDa band throughout the glycerol gradient fractions (FIG. 2A). After deglycosylation, the molecular weight drops to about 62 kDa (FIG. 3). Isolated human TNF α -con has a very similar apparent molecular weight of about 86.5 kDa (Fig. 4B), as shown by the correlation of enzyme activity with the 86.5 kDa band throughout the glycerol gradient fractions (FIG. 4A).

30

6.2. ISOLATION OF A PARTIAL cDNA SEQUENCE ENCODING PORCINE TNF α -CON

6.2.1. MATERIALS AND METHODS

N-terminal Sequencing

Affinity-purified material was either sequenced directly, or analyzed by SDS-PAGE using two 8-16% Novex (San Diego, CA) mini-gels (100 x 100 x 1mm) in Tris-glycine buffer. Electrophoretically separated proteins were detected using ISS Pro-Green (Natick, MA) according

to manufacturer's directions. A prominent band at about 85 kDa was excised and the protein was electroeluted directly onto a Hewlett-Packard C18 sequencing column, as described by Moyer et al., 1994, in: Crabb, J. (ed),

5 Techniques in Protein Chemistry, Vol. V, pp. 195-204, Academic Press, San Diego, CA. *In situ* reduction, alkylation, and digestion with Lys-con (Wako) were performed according to Burkhart et al., 1993, in: Angeletti, R. (ed), Techniques in Protein Chemistry Vol.

10 IV, pp. 399-406, Academic Press, San Diego, CA, except that 40% acetonitrile was used in the digestion buffer. After digestion, the column was placed in-line on the HPLC using a Hewlett-Packard G1007A column adapter coupled to a Hypersil ODS (0.8 x 300 mm, LC Packings).

15 No peptides were observed following a gradient of 0-80% acetonitrile in 0.1% trifluoroacetic acid (TFA). The sequencing column was subsequently removed from the column adapter and inserted in a Hewlett-Packard G1005S Protein Sequencing System with on-line PTH analysis. A

20 single sequence, as shown below, was obtained after 42 cycles from material bound to the sequencing support.

Val Gln Asp Val Ile Glu Arg Phe Trp Glu Phe Ile Asp
 Lys Leu Ser Ile Asn Thr Phe Gly Lys Phe Leu Ala Asp
 25 Asn Ile Val Gly Ser Val Leu Val Phe Ser Leu Met Leu
 Trp Ile (SEQ ID NO 7)

Cloning of Porcine TNF α -con

Degenerate and oppositely oriented

30 oligonucleotide PCR primers were designed based on the partial amino acid sequence of porcine TNF α -con determined above, specifically to amino acid sequence Val Gln Asp Val Ile Glu₆ (SEQ ID NO 11) and amino acid sequence Ala Asp Asn Ile Val Gly₃₀ (SEQ ID NO 12). Two

35 primers were synthesized from each orientation to reduce the sequence degeneracy. Thus, primer conv-1 has sequence 5'-GTI CA(A/G) GA(T/C) GT(A/G) AT(T/C/A) GA-3'

(SEQ ID NO 3); primer conv-2 has sequence 5'-GTI CA(A/G) GA(T/C) GT(T/C) AT(T/C/A) GA-3' (SEQ ID NO 4); primer conv-3 has sequence 5'-CC IAC (A/G/T)AT (A/G)TT (A/G)TC (T/C)GC-3' (SEQ ID NO 5); and primer conv-4 has sequence 5'-CC IAC (A/G/T)AT (A/G)TT (A/G)TC (A/G)GC-3' (SEQ ID NO 6). Primers conv-1 (SEQ ID NO 3) and conv-2 (SEQ ID NO 4) represent the 5' nucleotide sequences and the two primers only differ at one base position as shown above. Conv-3 (SEQ ID NO 5) and conv-4 (SEQ ID NO 6) are the 3' primers as shown.

Reverse transcriptase PCR was performed on porcine spleen poly (A+) RNA according to the manufacturer's protocol (Invitrogen cDNA cycle kit). Each of four PCRs used a pair of primers, one 5' primer i.e., either conv-1 (SEQ ID NO 3) or conv-2 (SEQ ID NO 4), and one 3' primer, i.e., either conv-3 (SEQ ID NO 5) or conv-4 (SEQ ID NO 6), at a final concentration of 2 mM each. The PCR mixture was cycled 35 times (one cycle = 45 sec at 94°C, 2 min at 55°C, and 3 min at 72°C), followed by electrophoresis on a 1.2% agarose gel. The expected 89 bp PCR fragment (SEQ ID NO 8) was obtained when primer conv-3 (SEQ ID NO 5) was used with primer conv-1 (SEQ ID NO 3) or conv-2 (SEQ ID NO 4) in the reaction. However, primer conv-4 (SEQ ID NO 6) together with conv-1 (SEQ ID NO 3) or conv-2 (SEQ ID NO 4) gave rise to a fragment of approximately 300 bp. The two PCR fragments thus obtained were made blunt-ended, subcloned into Bluescript II SK at the SmaI site, and subjected to DNA sequencing using the Taq dideoxy terminator method. DNA sequence analysis revealed that the 89 bp fragment (SEQ ID NO. 8) encoded an amino acid sequence (SEQ ID NO 15) identical to the known partial peptide sequence of porcine TNF α -con on which the primers were based, as shown below.

35

5' GTG CAG GAC GTC ATC GAG CGG TTC TGG GAG TTC ATT GAC
Val Gln Asp Val Ile Glu Arg Phe Trp Glu Phe Ile Asp

AAG CTG AGC ATC AAT ACT TTC GGG AAG TTC CTG GCA GAC
 Lys Leu Ser Ile Asn Thr Phe Gly Lys Phe Leu Ala Asp

AAC ATC GTC GG 3' (SEQ ID NO 8)

5 Asn Ile Val Gly (SEQ ID NO 15)

However, the 300 bp fragment was highly homologous to a human actin-binding protein (filamin) according to a sequence comparison with the GenBank database, indicating that a non-specific PCR fragment was amplified using primer conv-4 (SEQ ID NO 6).

The 89 bp fragment (SEQ ID NO 8) was used as a probe to screen a porcine spleen cDNA library constructed in λ gt10. The probe was labeled by random priming (BRL 15 kit) in the presence of a 32 P-dCTP.

A single-stranded oligonucleotide of 55 bp was also synthesized as a probe according to the sequence of the 89 bp fragment. The 55 bp oligomer was located between the 5' and 3' PCR primers as shown below:

20

G CCG TTC TGG GAG TTC ATT GAC AAG CTG AGC ATC AAT
 ACT TTC GGG AAG TTC CTG (SEQ ID NO 13)

The 55 bp oligomer was labeled by kinase-end labeling using γ^{32} P-ATP. Hybridization of the screening filters was performed in a 40% formamide buffer at 39°C, and the final wash was in 1 x SSC (55 bp oligomer probe) or 0.2 x SSC (89 bp probe) at 46°C.

30

6.2.2. RESULTS

The initial screening resulted in the isolation of four positive clones out of 2.5×10^5 recombinants. These four clones (pSC-1, pSC-2, pSC-3 and pSC-5) ranged from 1.1 kb to 2.3 kb in size and were sequenced after subcloning into the EcoRI site of Bluescript II SK. The clone pSC-3 was completely sequenced in both directions by subcloning the smaller restriction fragments into Bluescript II SK and

using flanking T3 and T7 sequences as sequencing primers. Sequence comparison showed that the four clones are overlapping. FIG. 5 shows a contiguous mapping of the entire length of 2,414 bases covered by the four clones. 5 Sequence analysis demonstrated that the 2,414 bp domain contained the coding sequence of the known 41 amino acid peptide sequence obtained from purified porcine spleen TNF α -con, but did not contain the coding region for the N-terminus of the porcine TNF-con. The conserved Zn²⁺-binding 10 motif of metalloproteinases was also lacking.

To obtain further 5' nucleotide sequence of porcine TNF-con, the 5' 120 bp EcoRI-PstI fragment of pSC-2 clone was isolated and used as a probe to rescreen the porcine spleen cDNA library. Three positive clones were 15 obtained. Sequence comparison indicated that only one clone, pSC-8, had the extended 5' sequence. This clone contained a 50 bp coding sequence 5' to the pSC-2 clone. The 50 bp region encodes the HELGH motif. The total 2,464 bp nucleotide sequence (SEQ ID NO 9) and the deduced amino 20 acid sequence (SEQ ID NO 10), which represents a partial sequence of porcine TNF α -con, are shown in FIG. 6.

6.3. ISOLATION OF A cDNA SEQUENCE ENCODING HUMAN TNF α -CON

25 6.3.1. MATERIALS AND METHODS

Two λ gt10 cDNA libraries constructed either from human leukocyte poly(A⁺) RNA (Clontech) or human monocyte poly(A⁺) RNA were used to screen for the full length human TNF α -con cDNA. The 120 bp EcoRI-PstI fragment and its 30 flanking 3' 690 bp PstI-BamHI fragment from the porcine pSC-2 clone were labeled by random priming (BRL kit) and used to screen both libraries. Replicate filters were hybridized in 50% formamide at 42°C, and the final wash was in 0.2 x SSC with 0.1% SDS at 55°C. Of approximately 2.5 x 35 10⁵ clones from each library, six of 14 positive clones (hc7, hc9, hc11, 3'#1, 3'#4, 3'#5), as shown in FIG. 7, were plaque-purified and sequenced.

To obtain the extreme 5' end of the cDNA, two different procedures were carried out, which produced equivalent results. In a first procedure, a 330 bp EcoRI-EcoRV DNA fragment corresponding to the 5' end of hc11 (FIG. 5 7) was labelled by random hexamer primer (Amersham), and used to screen the human monocyte library. Filters were hybridized in 50% formamide at 42°C and washed in 0.2 x SSC at 65°C. Seven positive clones were isolated after screening approximately 2.5×10^5 clones. Two of the seven 10 clones containing the longest 5' extensions, as shown in FIG. 7 (5'#4, 5'#7) were plaque-purified for further sequence analysis.

In a second procedure, a 5' RACE procedure was conducted using a RACE kit from Gibco/BRL, as described 15 below. See also Frohman et al., 1988, Proc. Natl. Acad. Sci. USA, 85:8998-9002, which is incorporated herein by reference.

First Strand cDNA Synthesis

20 Synthesis of a first strand was carried out using oligonucleotide primer RACE1 (22-mer) having the following sequence: 5'-CCTAGAGTCAGGCTCACCAACC-3' (SEQ ID NO 32), which is complementary to bp no. 541-520 of TNF α -con (FIG. 1) in sequence with Met start at bp no. 164-166. The following 25 were combined: 1 μ l RACE1 Oligonucleotide (2.5 pmoles/ μ l); 1 μ l Poly(A+) RNA from THP1-5A cells treated for 3 hr with TNF α and dibutyryl cyclic AMP (0.84 μ g); and 9.5 μ l DEPC-treated dH₂O. The mixture was incubated at 70°C for 10 min and then chilled on ice for 1 min.

30 The following was added to the above mixture: 2.5 μ l 10X reaction buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl]; 3.0 μ l 25 mM MgCl₂; 1.0 μ l 10 mM dNTPs; 2.5 μ l 0.1 M DTT; and 0.5 μ l RNasin (20 U). The solution was mixed and incubated at 42°C for 2 min. SUPERScript II™ reverse 35 transcriptase (1 μ l, 200 U, Gibco/BRL) was added and the mixture was incubated at 42°C for 30 min and then at 70°C for 15 min. RNase H (1 μ l, 2 U) was added, and the mixture

incubated at 55°C for 10 min, and then chilled on ice. The cDNA was purified using GLASSMAX™ DNA isolation cartridges according to manufacturers protocol [Gibco/BRL, catalog no. 18374-025]. Samples were partially dried in vacuo to bring 5 volume to approximately 30 µl.

TdT Tailing of cDNA

The following were combined in three separate equivalent reactions: 7.5 µl DEPC-treated H₂O; 2.5 µl 10X reaction buffer; 1.5 µl 25 mM MgCl₂; 2.5 µl 2 mM DCTP; and 10 µl of cDNA sample. The mixtures were incubated at 94°C for 2 min, then chilled on ice for 1 min, and 1 µl terminal deoxynucleotidyl transferase (10 U/µl) was added. The mixtures were then incubated at 37°C for 10 min, at 70°C for 15 min, and then kept on ice.

PCR of cDNA and Recovery

In each of 15 separate equivalent 50 µl reactions, the following were combined: 30 µl dH₂O; 4.0 µl 10X reaction buffer; 3.0 µl 25 mM MgCl₂; 1.0 µl 10 mM dNTPs; 1 µl RACE2B Oligonucleotide (10 pmoles/µl); and 1 µl Anchor Oligo-nucleotide (10 pmoles/µl).

RACE2B oligonucleotide (48-mer) has the following sequence (SEQ ID NO 33):

25 5'-CACGCGTCGACTAGTTACCATCCACCACCACGACCTTGAAATTTTGTG-3'

MluI _____ complementary to bp no. 467-435 in

SalI _____ sequence with Met start at bp no 164-166

SpeI;

Anchor Oligonucleotide, from Gibco/ BRL, has the 30 following sequence (SEQ ID NO 34):

5'-CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3'.

The reaction mixtures were incubated at 80°C for 5 min. Five µl of HOT TUB™ Polymerase (Amersham Inc.) in 1X reaction buffer (1 U/5µl) was added, and the mixtures cycled 35 in a Perkin Elmer 9600 thermal cycler 34 times (1 min at 94°C, 30 sec at 50°C, 2 min at 72°C); and 1 time (1 min at 94°C, 30 sec at 50°C, and 10 min at 72°C). All 15 reaction

mixtures were combined and the DNA was precipitated at -20°C with 2 M ammonium acetate and 2 volumes of 100% EtOH. DNA precipitate was collected by centrifugation, rinsed with cold 70% EtOH, dried *in vacuo*, and resuspended in 20 μl of 5 dH_2O .

DNA was electrophoresed through a 1.5% agarose gel in 0.5X TBE buffer. DNA migrating as a stained smear from approximately 375-700 bp was collected by electro-elution into a well containing 100 mM ammonium acetate. The DNA was
10 extracted once with phenol/chloroform (1:1), and precipitated at -20°C with 2 M ammonium acetate and 2 volumes of 100% EtOH. DNA precipitate was collected by centrifugation, rinsed with cold 70% EtOH, dried *in vacuo*, and resuspended in 21.5 μl of Dh_2O .

15

Restriction and Ligation of cDNA into Plasmid

10X H buffer (2.5 μl) (Boehringer Mannheim) and SpeI (1 μl ; 10 U/ml) were added to a DNA sample and incubated at 37°C for 2 hr. DNA was electrophoresed through
20 a 1.5% agarose gel in 0.5X TBE buffer. DNA migrating as a stained smear from approximately 200-800 bp was excised from the gel and recovered using a SPINBIND™ cartridge according to manufacturer's protocol (FMC Corp.), with an additional extraction with phenol/chloroform (1:1) after elution from
25 the cartridge. DNA was precipitated at -20°C with 2 M ammonium acetate and 2 volumes of 100% EtOH. DNA precipitate was collected by centrifugation, rinsed with cold 70% EtOH, dried *in vacuo*, and resuspended in 7.25 μl of dH_2O .

30

To the DNA was added pBS-SKII+ plasmid (Invitrogen) (1.0 μl , approx. 25 ng) which had been digested with SpeI and dephosphorylated with calf intestinal phosphatase. The mixture was incubated at 55°C for 2 min. The following was then added: 10X T4 Ligase Buffer (1 μl)
35 (Boehringer Mannheim); 0.5 μl 10 mM ATP; and 0.25 μl T4 Ligase (Boehringer Mannheim) (1 U/ μl); and the ligation mixture was incubated at 15°C for 7.5 hr.

Bacterial Transformation And Clone Characterization

DH5 α MAX EFFICIENCY™ Cells (Gibco/BRL) (100 μ l) were transformed with 1.5 μ l of the ligation mixture according to manufacturers protocol. Cells were plated onto 5 100 mm LB plates containing 50 μ g/ml ampicillin, overlaid with 75 μ l Blu-Gal and 10 μ l 100 mM IPTG, and incubated overnight at 37°C.

Plasmid DNA from white colonies was isolated by standard methods and analyzed for cDNA inserts by digestion 10 with SpeI and electrophoresis through a 1.0% agarose gel in 0.5X TBE buffer. Clones containing cDNA inserts were sequenced and positive clones were identified by comparison to the 5'-nucleotide sequence of TNFc clone hc11. The DNA sequence (SEQ ID NO 39) of the RACE14 clone is shown in FIG. 15 11.

Constructing A cDNA Encoding The Entire Open Reading Frame (ORF) Of TNF Convertase

Assembly Of RACE14 and hc-11

20 RACE14 cDNA (SEQ ID NO 39) (FIG. 11) was cloned into the SpeI site of Bluescript plasmid, pBS-SKII+ with the 3'-end of the cDNA oriented closest to the T7 promoter site on the vector. RACE14/pBS-SKII (3,485 bp) was cut with BglII and HindIII and the ends were dephosphorylated. hc-11 25 cDNA was cloned into the EcoRI site of Bluescript plasmid pBS-SKII+ with the 3'-end of the cDNA oriented closest to the T7 promoter site on the vector. hc-11/pBS-SKII (4,323 bp) was digested with BglII and HindIII to excise the hc-11 cDNA. The BglII/HindIII digested hc-11 cDNA was ligated 30 into the BglII/HindIII digested RACE14/pBS-SKII. The resulting plasmid (4,677 bp) was designated RACE14/hc11-pBS-SKII.

Assembly Of RACE14/hc-11 With ORF Of hc-7 And hc-9

35 RACE14/hc11-pBS-SKII was digested with EcoRI and NotI, purified from the excised piece of hc-11 cDNA and dephosphorylated. hc-7 cDNA (FIG. 7) was cloned into the

EcoRI site of Bluescript plasmid, pBS-SKII+ with the 3'-end of the cDNA oriented closest to the T3 promoter site on the vector. hc-7/pBS-SKII (4,813 bp) was cut with EaeI and NcoI. DNA fragments were separated on a 1% agarose, 0.5X TBE gel and the 750 bp EaeI/NcoI fragment of hc-7 was isolated using FMC SPINBIND™ cartridges as above. hc-9 cDNA (FIG. 7) was cloned into the EcoRI site in Bluescript plasmid pBS-SKII+ with the 3'-end of the cDNA oriented closest to the T7 promoter site on the vector. hc-9/pBS-SKII (4,895 bp) was cut with EaeI and EcoRI. DNA fragments were separated on a 0.7% agarose, 0.5X TBE gel and the 1,098 bp EaeI/EcoRI fragment of hc-9 was isolated using FMC SPINBIND™ cartridges as above. EcoRI/NotI digested RACE14/hc11-pBS-SKII was mixed with both the 750 bp EaeI/NcoI fragment of hc-7 and the 1,098 bp EaeI/EcoRI fragment of hc-9 and ligated together with T4 DNA ligase.

PCR Generation Of cDNA Encoding
The Entire ORF Of TNF α -Con

The ligation mix (10 μ l) of RACE14/hc11-pBS-SKII, 750 bp EaeI/NcoI fragment of hc-7 and the 1,098 bp EaeI/EcoRI fragment of hc-9 was subjected to PCR by combining the following: 1 μ l of the 10 μ l ligation mixture, 1 μ l of the 5' oligonucleotide primer - TNFC-4 (bp 164-190) (SEQ ID NO. 35) (100 pmoles/ml) (see below); 1 μ l of the 3' oligonucleotide primer - TNFC-3 (bp 2754-2719) (SEQ ID NO. 36) (100 pmoles/ml) (see below); 4.5 μ l 10X HOT TUB™ buffer (low magnesium; Amersham); 1 μ l 10 mM dNTPs; and 36.5 μ l dH₂O.

TNFC-4 oligonucleotide sequence (SEQ ID NO 35) is:
5'-CGGGATCCATGAGGCAGTCTCTCCTATTCCTGACC-3'

BamHI

bp no. 164-190 of TNF α -con cDNA.

TNFC-3 oligonucleotide sequence (SEQ ID NO 36) is:
5'-CAGGAAGTTGCGGCCGCTGACCAGCATCTGCTAAGTCACTTCCCAGTCTTCAC-3'

NotI

bp no. 2754-2719 of TNF α -con cDNA (FIG. 1).

The mixture was incubated at 80°C for 5 min, and 5 μ l (1 U) HOT TUB™ Polymerase in 1X buffer (Amersham) was then added. The mixture was cycled in a Perkin Elmer 9600 thermal cycler 25 times (1 min at 94°C, 2 min at 55°C, 2 min at 72°C), and 1 time (1 min at 94°C, 2 min at 55°C, 10 min at 72°C).

**Restriction Enzyme Digestion and
Ligation of cDNA Into Plasmid**

10 The PCR reaction mixture was extracted once with phenol/chloroform (1:1), and precipitated at -20°C with 2 M ammonium acetate and 2 volumes of 100% EtOH. DNA precipitate was collected by centrifugation, rinsed with cold 70% EtOH, dried *in vacuo*, resuspended in 1X NEB3 buffer
15 containing 100 μ g/ml BSA, 15 U NotI and 10 U BamHI, and incubated for 3 hr at 37°C. Digested DNA was electrophoresed through a 0.7% agarose gel in 0.5X TBE buffer, and DNA migrating at approximately 2,500 bp was excised from the gel and recovered using a SPINBIND™
20 cartridge as above, followed by an additional extraction with phenol/chloroform (1:1) after elution from the cartridge. The DNA was precipitated at -20°C with 2 M ammonium acetate and 2 volumes of 100% EtOH. DNA precipitate was collected by centrifugation, rinsed with
25 cold 70% EtOH, dried *in vacuo*, and resuspended in 10 μ l of dH₂O.

The following were combined in a first tube: 1.0 μ l of pBS-SKII+ plasmid (Invitrogen) (approx. 25 ng) previously digested with NotI and BamHI and dephosphorylated
30 with calf intestinal phosphatase; 1 μ l of digested PCR-generated cDNA from immediately above; and 6.25 μ l dH₂O.

The following were combined in a second tube: 1 μ l of pBS-SKII+ plasmid (Invitrogen) (approx. 25 ng) previously digested with NotI and BamHI and dephosphorylated
35 with calf intestinal phosphatase; 3.0 μ l of digested PCR-generated cDNA from immediately above; and 4.25 μ l dH₂O.

The mixture in each tube was incubated at 55°C for 2 min. To each tube was added: 1 µl of 10X T4 Ligase Buffer (Boehringer Mannheim); 0.5 µl 10 mM ATP; and 0.25 µl T4 Ligase (Boehringer Mannheim) (1 U/µl), and each tube was incubated at 15°C for 6 hr.

Bacterial Transformation And Clone Characterization

DH5α MAX EFFICIENCY™ cells (Gibco/BRL) (67 µl) were transformed with 1.5 µl each of the ligation mixtures from above according to manufacturers protocol. Cells were plated onto LB plates containing 50 µg/ml ampicillin, overlayed with 75 µl Blue-Gal and 10 µl 100 mM IPTG, and incubated overnight at 37°C. Plasmid DNA from "white" colonies was isolated by standard methods and analyzed for cDNA inserts by digestion with PstI, and duplicate aliquots were digested with BamHI and NcoI and electrophoresed through a 1.0% agarose gel in 0.5X TBE buffer. Four of 6 clones contained cDNA with the expected restriction enzyme digestion pattern. DNA from 2 clones was sequenced. DNA from one of the two clones, pBS/TNFC-1, was correctly assembled as determined by its DNA sequence. pBS/TNFC-1 corresponds to bp 164-2754 of the cDNA sequence encoding TNFα-con shown in FIG. 1, but with BamHI and NotI ends added to the sequence.

25

Expression Vector Construction

The full-length cDNA encoding the human TNFα-con was subcloned into a baculovirus expression vector, pFastBac1 (Gibco/BRL), as follows: pBS/TNFC-1 (5 µg) was digested with 1 µl of BamHI (Promega, 10 U/µl); 1 µl of NotI (Promega, 10 U/µl); 1 µl of PvuI (Gibco/BRL, 10 U/µl) (note: PvuI was added to further cut pBluescriptSK and facilitate band identification and isolation from gel); 3 µl of 10x New England Biolabs (NEB) restriction buffer no. 3; and adjusted to a final volume of 30 µl with water. The mixture was incubated for 2 hr at 37°C and then run on a 1% agarose-TAE gel.

The approx. 2.9 kb BamHI - NotI insert band on the gel was excised in a gel piece. The gel piece was frozen at -70°C for 15 min, incubated at 37°C for 15 min, placed inside the upper chamber of a Millipore spin filter unit 5 (ULTRAFREE™ Probind), and then centrifuged in a microcentrifuge (Eppendorf) for 10 min at maximum speed. The eluate collected at the bottom tube was saved. This material was ligated to pFastBac1 containing the polyhedrin promoter (pFBPH), opened at the multiple cloning site by 10 double digestion with BamHI and NotI, and purified as described above for pBS/TNFC-1. Several ligation reactions were set up at a fixed concentration (50 ng) of pFastBac1 and variable amounts of insert (0, 50 and 250 ng), in a final volume of 20 µl containing 2 µl of 10x T4 DNA ligase 15 buffer and 2 µl of T4 DNA ligase. The reaction was incubated at 12°C overnight.

Ligated material (10 µl) was used to transform 100 µl of DH5α MAX EFFICIENCY™ competent cells (Gibco/BRL) by calcium chloride precipitation according to supplier's 20 instructions. Transformation mixture (100 µl) was plated onto a 2x YT/agar plate containing 100 µg/ml ampicillin. The plate was incubated overnight at 37°C. Colonies were picked up from the plate and diluted in 2 ml of the same medium without agar. These cultures were incubated at 37°C 25 overnight with vigorous shaking. Plasmids were isolated using the WIZARD™ miniprep DNA purification kit (Promega), according to manufacturer's instructions. 5 µl of each isolated plasmid were incubated with 1 µl of 10X NEB buffer no. 4, 0.5 µl of NotI (Promega; 10 U/µl), and 0.5 µl of 30 BamHI (Promega; 10 U/µl), and adjusted to a final volume of 10 µl with water. After 1 hr of incubation at 37°C, samples were run in a 1% agarose-TAE gel, and one candidate with the correct restriction pattern was identified. A larger plasmid preparation was made out of this isolate (100 ml in 35 the same culture medium as described above), and the plasmid was extracted and purified using the Qiagen plasmid Midi kit, according to manufacturer's directions. Sequence of

the pure plasmid was confirmed by sequencing analysis at the Glaxo Wellcome Core DNA Sequencing Facility. This plasmid was designated pFBPH/TNFC.

An expression vector containing a partial TNF α -con 5 cDNA was also constructed in pFastBac, starting at codon 164 (Met) and ending at codon 651 (Arg). This region encodes a polypeptide that spans from before the catalytic domain to before the transmembrane domain. This construct was made as follows. The oligonucleotides M3 and M7 were used to obtain 10 the desired cDNA fragment from PBS/hc-7 by PCR:

M3: 5'-GCGCGCGCGCCATATGTTAGTTTATAAATCTGAAGATATCAAGAAT-GTTTCACG-3' (SEQ ID NO. 37);

M7: 5'-CGCGCGCGCGGGATCCCTATCGTTCAATTACATCCTGTAC-TCGTTTCTCAC-3' (SEQ ID NO. 38).

15 M3 and M7 were diluted after purification to a final concentration of 20 μ M in water. Each primer (1 μ l) was added to a tube containing 11.5 μ l of water, followed by the addition of 2 μ l of Stratagene OPTIPREP[™] buffer no. 3, 2 μ l of 10 mg/ml bovine serum albumin, 1 μ l (0.1 μ g) of 20 pBS/hc-7, 0.5 μ l of a 100 mM deoxynucleotides mixture (Stratagene), and 1 μ l of VENT[™] polymerase (New England Biolabs).

The reaction mixture was cycled in a Perkin-Elmer model 9600 thermal cycler using the following PCR protocol 25 for a total of 30 cycles: 30 sec at 94°C, 30 sec at 50°C, and 2 min at 72°C. After reaction completion, 2 μ l were run on a 1% agarose-TAE buffer to confirm product size and quality. The remaining material was diluted to 85 μ l with water, followed by the addition of 10 μ l of 10x NEB buffer 30 no. 4 and 5 μ l of BamHI (New England Biolabs, 20 U/ μ l). The restriction reaction was incubated overnight at 37°C, and the sample was resolved in a 1% agarose-TAE preparative gel. The DNA band corresponding to the restricted PCR product was excised, and the gel piece was purified by spin filtration 35 as described above.

The eluate collected from the bottom of the centrifuge tube was used in a ligation reaction to Pfastbac1

(Gibco/BRL) containing an inverted multiple cloning site cut with StuI and BamHI. as follows: 10 μ l (1 μ g) of PfastBac1 were mixed with 5 μ l of 10x NEB buffer no. 4, 2.5 μ l of Stu I (New England Biolabs, 10 U/ μ l), and 30 μ l of water. The 5 StuI cleavage reaction was allowed to proceed for 3 hr at 37°C, and then 2.5 μ l of BamHI (New England Biolabs, 20 U/ μ l) were added with further incubation at 37°C for 3 hr. The restricted plasmid was purified as described for the PCR product. Ligations were done in a final volume of 20 μ l, 10 and transformation and colony screening were done as described above. The ligation reaction consisted of 2 μ l (approx. 50 ng) of restricted Pfastbac1, 10 μ l (approx. 150 ng) of restricted PCR product, 2 μ l of 10x T4 DNA ligase buffer (Promega), and 2 μ l of T4 DNA ligase-HC (Promega). 15 The reaction was incubated at 12°C overnight.

Ligated material (10 μ l) was used to transform 100 μ l of DH5 α MAX EFFICIENCY™ competent cells (Gibco/BRL) by calcium chloride precipitation following the supplier's instructions. 100 μ l of the transformation mixture were 20 plated onto a 2x YT/agar plate containing 100 μ g/ml ampicillin. The plate was incubated overnight at 37°C. Colonies were picked up and DNA was extracted and purified as described above. 5 μ l of each isolated plasmid were incubated with 1 μ l of 10X NEB buffer no. 4, 0.5 μ l of NdeI 25 (New England Biolabs, 10 U/ μ l), and 0.5 μ l of BamHI (Promega, 10 U/ μ l), and adjusted to a final volume of 10 μ l with water. After 1 hr of incubation at 37°C, samples were run in a 1% agarose-TBE gel, and one candidate with the correct restriction pattern was identified. More plasmid 30 was prepared as described above. Sequence of pure plasmid (pFBN2) was confirmed by DNA sequencing analysis.

After obtaining pFBN2 it was determined that pBS/hc-7 contains a T to C base change at position 1,512 (FIG. 1B), resulting in a Phe to Ser change in the 35 translated amino acid sequence. This mutation was fixed by replacing the NcoI to XhoI fragment of pFBN2 (containing the mutation) with a NcoI to XhoI fragment from clone hc-11

(wild type T at position 1,512), in the multi-step procedure described below.

In the first step of the procedure, an additional XhoI site in pFBN2's multiple cloning site was outcloned. 5 2 μ l (4 μ g) of pFBN2 were incubated at 37°C for 3 hr with 2 μ l of NEB buffer no. 4, 1 μ l of NotI (Promega, 10 U/ μ l), 1 μ l of HindIII (Promega, 10 U/ μ l), and 14 μ l of water. T4 DNA polymerase (0.5 μ l) and 0.5 μ l of a dNTPs mixture (Stratagene, 100 mM each) were then added, and incubation 10 proceeded at 37°C for 1 hr. 2 μ l of 10X T4 DNA ligase buffer (Promega) and 2 μ l of T4 DNA ligase (Promega, HC) were added, and the mixture was incubated at 12°C for 6 hr. 200 μ l of *E. coli* Stbl-2 MAX EFFICIENCY™ competent cells (Gibco/BRL) were added to the tube, and the transformation 15 mixture was incubated on ice for 30 min, followed by a 30 sec pulse at 42°C and plating of the whole mixture on a 2x YT/agar plate containing 100 μ g/ml ampicillin. The plate was incubated for 24 hr, and colonies were picked and plasmid minipreps prepared as described above. Restriction 20 analysis was carried out using XhoI, HindIII, NotI and XhoI-AccI combined in order to confirm the loss of the HindIII, NotI and the extra XhoI site. One construct with the correct restriction patterns was identified, its plasmid DNA prepared as described before, and sequenced. This plasmid 25 was designated pFBN2/ Δ XhoI, and its sequence is identical to pFBN2, except for the deletion of the HindIII to NotI segment of the multiple cloning site.

In the second step of the procedure, both pFBN2/ Δ XhoI and pBS/hc-11 were digested with NcoI and XhoI 30 as follows. 10 μ g of either plasmid was mixed with 6 μ l of NEB buffer no. 4, 3 μ l of NcoI (Promega, 10 U/ μ l), 3 μ l of XhoI (Gibco/BRL, 10 U/ μ l), and adjusted to a final volume of 60 μ l with water. These mixtures were then incubated for 2 hr at 37°C, and loaded on a 1% agarose-TAE preparative gel. 35 The backbone fragment of pFBN2/ Δ XhoI and the approx. 380 bp fragment from pBS/hc-11 were excised and purified by spin-filtration as described above. 50 ng (4 μ l) of the backbone

fragment were mixed with 12 ng (12 μ l) of the small hc-11 fragment, 2 μ l of 10x T4 DNA ligase buffer (Promega) and 2 μ l of T4 DNA ligase (Promega, HC), and incubated at 12°C overnight. Stbl-2 MAX EFFICIENCY™ competent cells (200 μ l; 5 Gibco/BRL) were added, and the mixture was incubated at 0°C for 30 min, followed by a 30 sec pulse at 42°C and plating onto a 2x YT-agar plate containing 100 μ g/ml ampicillin. The plate was incubated at 30°C for 24 hr. Colonies were picked and plasmid minipreps were performed as described 10 above. Isolated plasmids were analyzed by double digestion with XhoI (Gibco/BRL, 10 U/ μ l) and AccI (New England Biolabs, 10 U/ μ l). One plasmid exhibited the correct restriction pattern, and the corresponding isolate was used for making a larger plasmid preparation as described above. 15 The plasmid thus obtained was sequenced in order to confirm the wild type sequence. This plasmid was designated pFBN2/hc-11, and its sequence is identical to that of pFBN2/ Δ XhoI, except for the presence of the wild type T residue at position 1,512.

20 In the third step of the procedure, pFBN2/hc-11 was used to repair pFBPH/TNFC. Both pFBN2/hc-11 (10 μ g) and pFBPH/TNFC (5 μ g) were mixed with 5 μ l of NEB buffer no. 4, 2.5 μ l of NsiI (New England Biolabs, 10 U/ μ l), 2.5 μ l of NcoI (Promega, 10 U/ μ l), and adjusted to 50 μ l with water. 25 Mixtures were incubated for 2 hr at 37°C, and the digests were resolved in a 1% agarose-TAE preparative gel. The backbone fragment of pFBPH/TNFC and the approx. 600 bp fragment from pFBN2/hc-11 were excised and purified as described above. 50 ng (2 μ l) of pFBPH/TNFC backbone 30 fragment were then mixed with 50 ng (10 μ l) of pFBN2/hc-11, 2 μ l of 10x T4 DNA ligase buffer (Promega), 2 μ l of T4 DNA ligase (Promega, HC) and 4 μ l of water. The reaction was incubated overnight at 12°C, and then 200 μ l of Stbl-2 competent cells were transformed with this ligate as 35 described above. Transformant colonies were isolated and the DNA purified and analyzed as described above by performing restriction digests with NcoI and NsiI. Three

correct isolates were worked up as described for sequence confirmation. The new full-length expression vector was designated pFBPH/TNFCA, and differs from pFBPH/TNFC by the presence of the correct T residue at position 1512.

5

6.3.2. RESULTS

Human TNF α -con is characterized by the cDNA sequence (SEQ ID NO 1) and deduced amino acid sequence (SEQ ID NO 2) shown in FIG. 1. The cDNA contains a 163 bp
10 untranslated region at the 5' end, followed by a protein coding region of 2,475 bp, and a 304 bp untranslated region at the 3' end. The first methionine residue (presented as the first amino acid residue in FIG. 1) is the initiation methionine, as indicated by the presence upstream of this
15 residue of a termination codon (TAG) at bases 62 to 64.

Full length TNF α -con comprises 824 amino acids. TNF α -con begins with amino acid Met and ends with amino acid Cys. Based on the deduced amino acid sequence, the predicted molecular weight of the protein is 93.02 kDa.
20 Hydropathy plots reveal 2 hydrophobic segments representing a putative signal peptide at amino acid residues 1-17, and a transmembrane region at amino acid residues 672-691. A search of GeneBank identified the following motifs that are unique to the metalloproteinase family: cysteine switch
25 motif (aa 181-185), zinc-binding motif (aa 405-409), and Met-turn motif (aa 435-437).

7. EXAMPLE: PREPARATION OF A BIOTINYLATED INHIBITOR OF TNF α -CON

30

A biotinylated inhibitor of TNF α -con, useful for the affinity purification of TNF α -con as described above (Section 6.1.1), was prepared as follows.

Unless otherwise noted, chemicals were obtained
35 from commercial suppliers and used without further purification. Thin layer chromatography (TLC) analyses were performed with Merck 60 F₂₅₄ 0.25 micron silica gel plates.

Flash column chromatography was performed using 230-400 mesh silica gel (EM Science). Compound homogeneity was determined by analytical reverse-phase HPLC using a Dynamax-60A column with eluants A (water, 0.1% TFA) and B (acetonitrile, 0.1% TFA) with gradient elution from 85% A:15% B to 20% A:80% B over 30 min with a flow rate of 1.5 ml per min. Compound purification was carried out by preparative reverse-phase HPLC using a 2-in. diameter Dynamax-60A column using the same eluants as described for analytical HPLC with an appropriately varied gradient at a flow rate of 45 ml per min. ^1H NMR spectra were obtained with a Varian Unity 300 with line broadening of 0.5 Hz and a relaxation delay of 0.1 seconds. Proton-decoupled ^{13}C spectra were obtained at 75 MHz using the same instrument. Chemical shifts are reported in ppm. Low resolution mass spectra were performed on a JEOL AX505 using fast atom bombardment (FAB) using thioglycerol, 3-nitrobenzyl alcohol, or 3-nitrobenzyl alcohol/lithium acetate as the matrix solvent.

20

Preparation of (A)

To a solution of CBZ-phenylalanine (5.57 g, 0.0192 mol) in CH_2Cl_2 (70 ml) at 25°C were added HOBt $\cdot\text{H}_2\text{O}$ (2.94 g, 0.0192 mol), BOC-1,6-hexanediamine (5.0 g, 0.0198 mol) and Et_3N (2.97 ml, 0.0213 mol). The solution was cooled to 0°C. To the solution was added dicyclohexylcarbodiimide (4.31g, 0.0209 mol), and the solution was stirred and allowed to warm to 25°C over 16 hr. The mixture was filtered, the filtrate was washed using dilute HCl, and the layers were separated. The organic layer was washed using saturated, aqueous NaHCO_3 and the layers were separated. The organic layer was dried (MgSO_4) and filtered, and the filtrate was concentrated to afford 9.53 g (99%) of product (A) (FIG. 9A). This material was used in the next reaction without further purification. R_f 0.42 (30% $\text{EtOAc}:\text{CH}_2\text{Cl}_2$); ^1H NMR (300 MHz, CDCl_3) δ 1.00 - 1.40 (m, 8H), 1.42 (s, 9H), 2.9 - 3.2 (m, 6H), 4.35 (q, 1H, $J = 7$ Hz), 4.55 (br s, 1H), 5.08

(s, 2H), 5.44 (br s, 1H), 5.78 (br s, 1H), 7.10 - 7.40 (m, 10H).

Preparation of (B)

5 To a solution of product (A) (5.00 g, 0.0100 mol) in EtOH (100 ml), H₂O (1.0 ml), and EtOAc (40 ml) was added 10% Pd/C (1.00 g) and the reaction flask was evacuated and purged using nitrogen. The reaction flask was evacuated and purged using hydrogen and the mixture was stirred at 25°C
10 under 1 atm of hydrogen for 6 hr. The reaction flask was evacuated and purged using nitrogen, the mixture was filtered, and the filtrate was concentrated to afford 3.22 g (88%) of product (B) (FIG. 9B). This material was used in the next reaction without further purification. ¹H NMR (300
15 MHz, CDCl₃) δ 1.2 - 1.6 (m, 17H), 2.6 - 2.8 (m, 1H), 3.0 - 3.3 (m, 4H), 3.58 - 3.62 (m, 1H), 4.55 (br s, 1H), 7.2 - 7.4 (m, 5H).

Preparation of (C)

20 Reagent (C) (FIG. 9C) was prepared by the following procedure. To a solution of concentrated sulfuric acid (75 ml) in distilled water (350 ml) were added D-leucine (FIG. 10A) (50.0 g, 0.381 mol) and potassium bromide (158 g, 1.33 mol), and the solution was cooled to just below
25 0°C. To the solution was added sodium nitrite (34.8 g, 0.504 mol) as a solution in distilled water (100 ml), dropwise, over a period of 1 hr. After the addition was complete, the mixture was allowed to stir at 0°C for 1 hr. Dichloromethane was added to the solution and the mixture
30 was stirred for several minutes. The layers were separated and the aqueous layer was extracted using dichloromethane. The layers were separated and the combined organic layer was dried (MgSO₄) and filtered, and the filtrate was concentrated to afford 45 g (61%) of the crude product.
35 This material was used in the next reaction without further purification: ¹H NMR (300 MHz, CDCl₃) δ 0.92-0.96 (m, 6H), 1.7-1.85 (m, 1H), 1.90-1.95 (m, 2H), 4.28 (t, 1H, J=7.6 Hz).

To a cold (-78°C) solution of the bromo-acid (45.6 g, 0.233 mol), produced above, in dichloromethane (200 ml) was introduced isobutene (200 ml) using a cold finger (-78°C). To the solution was added concentrated sulfuric acid (1.5 ml), dropwise, and the solution was allowed to stir and to warm to 25°C over 17 hr. The solution was concentrated to one-half of the original volume by removing the solvent under reduced pressure. The resulting solution was washed using 10% aqueous NaHCO₃ (2 x 200 ml) and the layers were separated. The organic layer was dried (MgSO₄) and filtered and the filtrate was concentrated under reduced pressure to afford 47 g (81%) of the crude product (FIG. 10B). This material was used in the next reaction without further purification: ¹H NMR (300 MHz, CDCl₃) δ 0.85-0.95 (m, 6H), 1.43 (s, 9H), 1.6-1.8 (m, 1H), 1.80-1.85 (m, 2H), 4.13 (t, 1H, J=7.6 Hz).

To a solution of dibenzyl malonate (46.5 g, 0.186 mol) in DMF (80 ml) at 25°C was added potassium tert-butoxide (20.7 g, 0.184 mol), portionwise, and with cooling (occasional icebath). After formation of a homogeneous solution, the solution was cooled (0°C) and the bromo-ester (4.77 g, 0.189 mol) was added dropwise as a solution in DMF (80 ml). After the addition was complete, the solution was allowed to stir at 5°C for 4 days. The mixture was partitioned between ethyl acetate and saturated aqueous ammonium chloride and the layers were separated. The aqueous layer was extracted using ethyl acetate and the layers were separated. The combined organic layer was concentrated under reduced pressure and the residue was dissolved in diethyl ether and was washed using brine. The layers were separated and the organic layer was dried (MgSO₄) and filtered and the filtrate was concentrated. The residue was purified using flash column chromatography (gradient elution using 97.5% hexane:ethyl acetate to 90% hexane:ethyl acetate) on 230-400 mesh silica gel to afford 50 g (60%) of the product (FIG. 10C). ¹H NMR (300 MHz, CDCl₃) δ 0.80-0.90 (m, 6H), 1.0-1.1 (m, 1H), 1.40 (s, 9H),

1.45-1.60, (m, 2H), 3.0-3.1 (m, 1H), 3.75, (d, 1H, $J=12$ Hz), 5.0-5.2 (m, 4H), 7.2-7.4 (m, 10H); MS (positive ion FAB) $m/z=455$ ($[M+H]^+$).

To the *tert*-butyl ester (34.2 g, 0.0753 mol) was added a solution (95:5) of TFA:H₂O (52.5 ml) and the solution was stored at 0°C for 12 hr. The TFA was removed under reduced pressure and the residue was diluted using dichloromethane. The solution was washed using brine and the layers were separated. The organic layer was dried (MgSO₄) and filtered and the filtrate was concentrated to afford 30 g (100%) of the crude product (FIG. 10D, also shown as C in FIG 9). This material was used in the next reaction without further purification: ¹H NMR (300 MHz, CDCl₃) δ 0.80-0.85 (m, 6H), 1.1-1.2 (m, 1H), 1.5-1.7 (m, 2H), 3.10-3.25 (m, 1H), 3.8, (d, 1H, $J=11$ Hz), 5.0-5.2 (m, 4H), 7.2-7.4 (m, 10H), 10.0-10.2 (br s, 1H).

Preparation of (D)

To a solution of the acid (C) (FIG. 9C) (3.10 g, 0.077 mol) in DMF (20 ml) were added HOBT•H₂O (1.36 g, 0.00886 mol), 4-methylmorpholine (0.947 ml, 0.00886 mol), and product (B) (FIG. 9B) (3.22 g, 0.00886 mol) as a solution in THF (20 ml), and the solution was stirred at 25°C for 16 hr. The mixture was filtered and the filtrate was concentrated under reduced pressure to afford an oily residue. The residue was dissolved in EtOAc, washed using aqueous 10% citric acid solution, and the layers separated. The organic layer was washed using an aqueous solution of 10% NaHCO₃ and the layers were separated. The organic layer was washed using a saturated aqueous solution of NaCl and the layers were separated. The organic layer was dried (MgSO₄) and filtered, and the filtrate was concentrated. The residue was purified using flash column chromatography (gradient elution using 100% CH₂Cl₂ to 30% Et₂O:CH₂Cl₂) on 230-400 mesh silica gel to afford 3.70 g (64%) of the major diastereomer (D) (FIG. 9D) and 1.25 g (20%) of the minor diastereomer. ¹H NMR data is reported for the major

diastereomer only. ^1H NMR (300 MHz, CDCl_3) δ 0.74 (d, 3H, J = 2.7 Hz), 0.76 (d, 3H, 2.7 Hz), 1.0 - 1.5 (m, 11H), 1.40 (s, 9H), 2.8 - 3.2 (m, 6H), 3.8 (d, 1H, J = 9.3 Hz), 4.4 - 4.6 (m, 2H), 5.0 - 5.2 (m, 4H), 5.7 (t, 1H, J = 6 Hz), 6.58 (d, 1H, J = 7.5 Hz), 7.2 - 7.4 (m, 15H).

Preparation of (E)

To a solution of (D) (FIG. 9D) (2.89 g, 0.00389 mol) in EtOH (21 ml) was added ammonium formate (1.23 g, 0.0195 mol). To the mixture was added 10% Pd/C (578 mg) as a slurry in isopropanol (5.25 ml), and the mixture was stirred at 25°C for 45 min. The catalyst was removed by filtration, the filtrate was treated with piperidine (0.423 ml, 0.00428 mol), and the solution was stirred at 25°C for 15 min before the addition of aqueous 37% formaldehyde (2.00 ml, 0.0245 mol). After the solution had stirred at 25°C for 19 hr, the solution was warmed to reflux temperature and was stirred for 1 hr. The solution was allowed to cool to 25°C, the solvent was removed under reduced pressure, and the residue was dissolved in EtOAc and was acidified using aqueous 10% citric acid solution. The layers were separated and the organic layer was washed using aqueous 1% K_2CO_3 . The layers were separated, and the aqueous layer was acidified to pH 4 using 6N HCl and was extracted using CH_2Cl_2 . The organic layer was dried (MgSO_4) and filtered, and the filtrate was concentrated. The residue was purified using flash column chromatography (elution using 25% MeOH:EtOAc) to afford 1.15 g (56%) of product (E) (FIG 9E). ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 0.73 (d, 3H, J = 6 Hz), 0.78 (d, 3H, J = 6 Hz), 1.0 - 1.6 (m, 11H), 1.4 (s, 9H), 2.6 - 3.0 (m, 6H), 3.3 - 3.4 (m, 1H), 4.35 (q, 1H, J = 6 Hz), 5.10 (s, 1H), 5.80 (s, 1H), 6.78 (t, 1H, J = 5.3 Hz), 7.0 - 7.2 (m, 5H), 7.80 (t, 1H, J = 5.2 Hz), 8.35 (br s, 1H); MS (positive ion FAB) m/z = 538 ($[\text{M}+\text{Li}]^+$).

Preparation of (F)

To the α,β -unsaturated acid (E) (1.15 g, 0.00216 mol) was added thiophenol (7.32 ml, 0.0714 mol) and the mixture was stirred in the dark at 60°C for 1 day. The solution was allowed to cool to 25°C and Et₂O was added to precipitate the product. The product was collected by filtration, the solid was washed using ether, and was dried under vacuum to afford 0.940 g (65%) of a 3.5:1 mixture of the diastereomeric products (F) (FIG. 9F). This crude mixture of diastereomers was used in the next reaction without further purification. ¹H NMR (300 MHz, DMSO-d₆) δ 0.70 (d, 3H, J = 6 Hz), 0.78 (d, 3H, J = 6 Hz), 1.1 - 1.5 (m, 20H), 2.2 - 3.2 (m, 10H), 4.5 - 4.6 (m, 1H), 6.75 (t, 1H, J = 5.4 Hz), 6.9 - 7.3 (m, 10H), 7.92 (t, 1H, J = 5.3 Hz), 8.40 (d, 1H, J = 8.5 Hz).

Preparation of (G)

To the acid (F) (400 mg, 0.623 mmol) in CH₂Cl₂ (3.0 ml) and DMF (0.76 ml) was added HOBt•H₂O (114 mg, 0.748 mmol) and the mixture was cooled to 0°C, whereupon WSCDI (143 mg, 0.748 mmol) and 4-methylmorpholine (82 ml, 0.748 mmol) were added. The mixture was stirred for 1 hr at 0°C to ensure complete formation of the activated ester. Hydroxylamine hydrochloride (65 mg, 0.934 mmol) and 4-methylmorpholine (103 ml, 0.934 mmol) were added as a solution in DMF (2.0 ml) dropwise, and the mixture was stirred for 1 hr. The solution was poured into a mixture of H₂O (7.5 ml), Et₂O (7.5 ml), and hexane (7.5 ml), and the product precipitated. The precipitate was collected by filtration, and the solid was washed using hexane and was dried under vacuum to afford 204 mg (50%) of the diastereomeric products (G) (FIG. 9G). The diastereomers were separated using RP HPLC to afford 160 mg (39%) of the major diastereomer. Analytical data are reported for the major diastereomer only. ¹H NMR (300 MHz, DMSO-d₆) δ 0.71 (d, 3H, J = 6.3 Hz), 0.78 (d, 3H, J = 6.3 Hz), 1.1 - 1.5 (m, 11H), 1.3 (s, 9H), 2.0 - 3.0 (m, 10H), 4.62 (1H), 6.75 (t,

1H, $J = 5.4$ Hz), 6.9 - 7.3 (m, 10H), 7.82 (t, 1H, $J = 5.3$ Hz), 8.38 (d, 1H, $J = 8.8$ Hz), 8.92 (br s, 1H), 10.44 (s, 1H); ^{13}C NMR (75 MHz, DMSO- d_6) δ 21.5, 24.1, 25.0, 25.9, 26.0, 28.1, 28.2, 29.0, 29.4, 32.1, 37.5, 45.7, 46.1, 54.0, 57.29, 125.09, 126.29, 127.06, 127.86, 128.77, 129.08, 136.57, 137.94, 155.64, 168.22, 170.87, 172.73; MS (positive ion FAB) m/z 657 ($[\text{M}+\text{H}]^+$).

Preparation of (H)

10 To the major diastereomer (G) (160 mg, 0.244 mmol) was added TFA (2.5 ml) and the solution was stirred in a tightly stoppered flask for 20 min. The TFA was removed under reduced pressure and the residue was purified using RP HPLC to afford, after lyophilization, 125 mg (77%) of the
15 TFA salt (H) (FIG. 9H), referred to hereinafter as GI 193463A. ^1H NMR (300 MHz, DMSO- d_6) δ 0.72 (d, 3H, $J = 6.4$ Hz), 0.79 (d, 3H, $J = 6.4$ Hz), 1.1 - 1.6 (m, 10H), 2.0 - 3.2 (m, 10H), 4.6 (m, 1H), 6.8 - 7.3 (m, 10H), 7.6 (br s, 3H), 7.86 (t, 1H, $J = 5.4$ Hz), 8.37 (d, 1H, $J = 8.8$ Hz), 8.83,
20 (s, 1H), 10.44 (s, 1H); MS (positive ion FAB) m/z 557 ($[\text{M}+\text{H}]^+$).

Preparation of (I)

The biotin derivative of GI 193463A (FIG. 9I), was
25 prepared as follows. GI 193463A (20 mg) was dissolved in 0.6 ml dimethyl formamide and treated with 9 μl triethylamine and 9 mg immunopure NHS-SS biotin (Pierce). After stirring overnight at rm temp, 2 ml water was added and the resulting solid was collected and purified by thin
30 layer chromatography (silica gel) eluted with 10% methanol in dichloromethane to yield 3 mg of final product.

8. EXAMPLE: SUBSTRATE SPECIFICITY OF A PARTIALLY PURIFIED HUMAN TNF α -CON

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Experiments were carried out to determine the substrate specificity of a partially purified human TNF α -

con. Partially purified human TNF α -con was generated from microsomal fractions containing convertase activity as follows.

Mono Mac 6 cells (Ziegler-Heitbrook et al., 1988, 5 Int. J. Cancer, 41:456-461) were grown in RPMI 1640 medium containing 10% fetal bovine serum, 0.1% pluronic, penicillin/streptomycin (50 units/ml of each), and L-glutamine (2 mM). Cells (8.2×10^{10}) were sedimented, washed in 50 mM HEPES buffer, pH 7.5, containing 0.25 M sucrose and 2 mM MgCl₂, and sedimented. Pellet (260 ml) was resuspended to 600 ml in cold wash buffer containing protease inhibitors (1 mM AEBSF, 10 μ M E-64, 10 μ M leupeptin, 1 μ M pepstatin, 10 μ M phosphoramidon, and 50 μ M DCI). The suspension was pressurized under 1,000 psi N₂ in a cavitator for 30 min with stirring at 4°C. Pressure was released over 1-2 min with collection of approx. 90% of the lysate. Cell breakage was greater than 80% as determined by trypan blue exclusion.

The broken cell suspension was sedimented in a GS-3 rotor at 3,500 rpm for 10 min. The supernatant (400 ml) was centrifuged in a TFA 20.25 rotor at 20,000 rpm for 45 min at 4°C. The resulting pellet (50 ml) was resuspended to a total volume of 750 ml in 10 mM HEPES buffer, pH 7.5, containing 0.2 M NaCl, 1.2% NP-40, and protease inhibitors as above, except DCI, via dounce homogenization and stirring for 30 min at 4°C. The resuspended material was then centrifuged in a TFA 20.25 rotor at 20,000 rpm for 45 min at 4°C. The resulting pellet had a volume of less than 10 ml.

The supernatant (approx. 750 ml) was passed at 10 ml/min over a column containing 100 ml packed conA-sepharose (Pharmacia) which had been previously equilibrated with 10 mM HEPES buffer, pH 7.5, containing 0.2 M NaCl and 1% NP-40. The loaded column was washed with 200 ml of equilibration buffer, followed by 800 ml of the same buffer but without NaCl. Material was retained overnight at 4°C on the conA column.

Protein was eluted from the conA column by passing 850 ml of 10 mM HEPES buffer, pH 7.5, containing 1% NP-40, 0.25 M methyl- α -D-mannopyranoside, and protease inhibitors as above, except DCI, over the column at 10 ml/min. The 5 eluate was collected and passed over a column containing 2 ml packed POROS™ HQ anion exchanger at 5 ml/min which had been previously equilibrated with 10 mM HEPES buffer, pH 7.5, containing 1% NP-40. The column was washed with 10 ml of equilibration buffer. The buffer level over the packed 10 bed was lowered to just cover the matrix and the inlet tube was flushed with 10 mM HEPES buffer, pH 7.5, containing 0.5 M NaCl, 1 % NP-40, and protease inhibitors as above, except DCI (high salt buffer). Several milliliters of high salt 15 inlet was reconnected. High salt buffer was passed over the column at 1 ml/min, and ten 1 ml fractions were collected. Fractions 2 and 3, which had a distinct coloration, were pooled and contained 15.4 mg/ml protein (Micro BCA Kit; BioRad), as well as considerable TNF α -con activity.

20 To exchange the detergent from NP-40 to dodecylmaltoside (ddm), the enzyme was desalted by dialysis in 10 mM HEPES, pH 7.5, 1% ddm and protease inhibitors. The enzyme was then loaded onto a 200 μ l POROS™ HQ column, and eluted off with the above buffer containing 0.5 M NaCl. 25 Fractions containing convertase activity were used in substrate mapping experiments.

Synthetic peptides were prepared that had substitutions at P1' (Val) or P2' (Arg) in the following substrate (SEQ ID NO 16):

30

Biotin-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Lys-(DNP)-NH₂
P4 P3 P2 P1 P1' P2' P3' P4' P5'

One thousand peptides were assembled in one 35 hundred pools of ten peptides each, via the Fmoc solid phase strategy (Atherton et al., 1989, Solid phase synthesis: a practical approach, IRL Press, Oxford). Assembly was

performed on 5 grams Rink Amide resin (NovaBiochem, lot no. A12697, cat. no. 01-64-0013, 0.46 mmol/gm) by a combination of manual and automated synthesis techniques. Assembly from the C-terminus to P3' (Ser) was performed batchwise in a 5 reaction shaker. The resin was transferred to an Advanced ChemTech 357 MPS for splitting to twenty reaction vessels, coupling of P2', recombining and splitting to two 96-reaction vessel blocks for completion of assembly and subsequent cleavage on an Advanced ChemTech 496 MOS.

10 An aliquot of substrate was removed and added to an enzyme preparation to a final concentration for each substrate of 1 μ M. The fluorescent substrate, NBD-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Lys(DMC)-Ser-Arg-NH₂ (SEQ ID NO 17) (10 μ M) was added to follow the reaction by monitoring 15 fluorescence at 370 Nm (excitation), 460 Nm (emission). In addition, cleavage of substrates could be compared to the standard substrate, Biotin-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Lys-(DNP)-NH₂ (SEQ ID NO 16), which was included at 1 μ M. The substrates were incubated with enzyme preparation at 20 37°C for 0.5-4 hr and the reaction quenched by adding an equal volume of 1% HFBA. Samples were passed over a POROS™ avidin column and the products subjected to LC/MS to determine relative intensities of components released in the reaction mixture. The relative reactivities of peptides 25 modified at P1' and P2' are shown in Table 1 below.

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Table 1Substrate Specificity Of A Partially Purified Human TNF α -Con

	Substrate	Relative V/K
5	Biotin-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser- Lys-(DNP)-NH ₂ (SEQ ID NO 18)	1
	Biotin-Leu-Ala-Gln-Ala-Phenylgly-Ala-Ser- Ser-Lys-(DNP)-NH ₂ (SEQ ID NO 19)	1.7
10	Biotin-Leu-Ala-Gln-Ala-HomoPhe-Ala-Ser- Ser-Lys-(DNP)-NH ₂ (SEQ ID NO 20)	1.6
	Biotin-Leu-Ala-Gln-Ala-Phenylgly-Arg-Ser- Ser-Lys-(DNP)-NH ₂ (SEQ ID NO 21)	1.5
15	Biotin-Leu-Ala-Gln-Ala-3-(3-pyridyl)-Ala- His-Ser-Ser-Lys-(DNP)-NH ₂ (SEQ ID NO 22)	0.5
	Biotin-Leu-Ala-Gln-Ala-NorLeu-Arg-Ser-Ser- Lys-(DNP)-NH ₂ (SEQ ID NO 23)	1.3
20	Biotin-Leu-Ala-Gln-Ala-NorLeu-Ala-Ser-Ser- Lys-(DNP)-NH ₂ (SEQ ID NO 24)	0.7

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As can be seen from the data, substrates substituted with natural or unnatural amino acids at P1' or P2' are recognized and cleaved by partially purified human TNF α -con.

5 A second set of experiments were conducted to determine the effect of substrate length on the proteolytic ability of a partially purified human TNF α -con. The DNP-labeled substrates listed in Table 2 below were obtained from Zeneca/Cambridge Research Biochemicals. In individual
10 reactions, substrates at a concentration of 10 μ M were reacted with a microsomal enzyme preparation, in RB buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 μ M ZnCl₂ and 2 mM CaCl₂) containing 10 μ M leupeptin. Control reactions were run with 10 μ M of a hydroxamate inhibitor of TNF α -con (GI 129471X) to
15 insure that products produced in test reactions were generated as a result of TNF α -con activity. Samples were run from 0.5-4 hr at 37°C, and quenched by adding an equal volume of 1% HFBA. Samples were chromatographed on a C18 Vydac column with a 0.1% HFBA water/acetonitrile gradient
20 from 22% to 36% acetonitrile. Product was monitored by measuring absorbance at 380 nm. Relative activities, V/K, for various sized substrates are shown in Table 2 below. The results show that if Ser-Pro-Leu is removed, the peptide cannot serve as a substrate, whereas truncations at the
25 carboxy terminus can take place up to P4' without a substantial loss in cleavability.

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Table 2

Cleavage of Various Sized Substrates
By A Partially Purified Human TNF α -Con

5	Substrates	Relative V/K
	DNP-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-NH ₂ (SEQ ID NO 25)	1
10	DNP-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-NH ₂ (SEQ ID NO 26)	0.6
	DNP-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-NH ₂ (SEQ ID NO 27)	0.5
15	DNP-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-NH ₂ (SEQ ID NO 28)	0.6
	DNP-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-NH ₂ (SEQ ID NO 29)	1.5
20	DNP-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-NH ₂ (SEQ ID NO 30)	1
	DNP-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-NH ₂ (SEQ ID NO 31)	no turnover

25

All patents, patent applications, and publications cited above are incorporated herein by reference in their entirety.

30 The present invention is not to be limited in scope by the specific embodiments described, which are intended as single illustrations of individual aspects of the invention. Functionally equivalent compositions and methods are within the scope of the invention. Indeed,
35 various modifications of the invention, in addition to those shown and described herein, will become apparent to those skilled in the field of molecular biology, medicine or

related fields from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: X, Inventor
Y, Inventor
Z, Inventor
- (ii) TITLE OF INVENTION: TUMOR NECROSIS FACTOR ALPHA CONVERTASE
- (iii) NUMBER OF SEQUENCES: 39
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Glaxo Wellcome
 - (B) STREET: Five Moore Drive
 - (C) CITY: Research Triangle Park
 - (D) STATE: North Carolina
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 27709
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: To Be Assigned
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Dadswell, Charles E.
 - (B) REGISTRATION NUMBER: 35,851
 - (C) REFERENCE/DOCKET NUMBER:
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (919) 990-6983
 - (B) TELEFAX: (919) 248-7988
 - (C) TELEX: 802813

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2942 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 164..2635

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGAGCCTGGC GGTAGAATCT TCCAGTAGG CGGCGCGGGA GGGAAAAGAG GATTGAGGGG	60
CTAGGCCGGG CGGATCCCGT CCTCCCCCGA TGTGAGCAGT TTTCCGAAAC CCCGTCAGGC	120
GAAGGCTGCC CAGAGAGGTG GAGTCGGTAG CGGGGCCGGG AAC ATG AGG CAG TCT	175
Met Arg Gln Ser	

1

CTC CTA TTC CTG ACC AGC GTG GTT CCT TTC GTG CTG GCG CCG CGA CCT Leu Leu Phe Leu Thr Ser Val Val Pro Phe Val Leu Ala Pro Arg Pro 5 10 15 20	223
CCG GAT GAC CCG GGC TTC GGC CCC CAC CAG AGA CTC GAG AAG CTT GAT Pro Asp Asp Pro Gly Phe Gly Pro His Gln Arg Leu Glu Lys Leu Asp 25 30 35	271
TCT TTG CTC TCA GAC TAC GAT ATT CTC TCT TTA TCT AAT ATC CAG CAG Ser Leu Leu Ser Asp Tyr Asp Ile Leu Ser Leu Ser Asn Ile Gln Gln 40 45 50	319
CAT TCG GTA AGA AAA AGA GAT CTA CAG ACT TCA ACA CAT GTA GAA ACA His Ser Val Arg Lys Arg Asp Leu Gln Thr Ser Thr His Val Glu Thr 55 60 65	367
CTA CTA ACT TTT TCA GCT TTG AAA AGG CAT TTT AAA TTA TAC CTG ACA Leu Leu Thr Phe Ser Ala Leu Lys Arg His Phe Lys Leu Tyr Leu Thr 70 75 80	415
TCA AGT ACT GAA CGT TTT TCA CAA AAT TTC AAG GTC GTG GTG GTG GAT Ser Ser Thr Glu Arg Phe Ser Gln Asn Phe Lys Val Val Val Val Asp 85 90 95 100	463
GGT AAA AAC GAA AGC GAG TAC ACT GTA AAA TGG CAG GAC TTC TTC ACT Gly Lys Asn Glu Ser Glu Tyr Thr Val Lys Trp Gln Asp Phe Phe Thr 105 110 115	511
GGA CAC GTG GTT GGT GAG CCT GAC TCT AGG GTT CTA GCC CAC ATA AGA Gly His Val Val Gly Glu Pro Asp Ser Arg Val Leu Ala His Ile Arg 120 125 130	559
GAT GAT GAT GTT ATA ATC AGA ATC AAC ACA GAT GGG GCC GAA TAT AAC Asp Asp Asp Val Ile Ile Arg Ile Asn Thr Asp Gly Ala Glu Tyr Asn 135 140 145	607
ATA GAG CCA CTT TGG AGA TTT GTT AAT GAT ACC AAA GAC AAA AGA ATG Ile Glu Pro Leu Trp Arg Phe Val Asn Asp Thr Lys Asp Lys Arg Met 150 155 160	655
TTA GTT TAT AAA TCT GAA GAT ATC AAG AAT GTT TCA CGT TTG CAG TCT Leu Val Tyr Lys Ser Glu Asp Ile Lys Asn Val Ser Arg Leu Gln Ser 165 170 175 180	703
CCA AAA GTG TGT GGT TAT TTA AAA GTG GAT AAT GAA GAG TTG CTC CCA Pro Lys Val Cys Gly Tyr Leu Lys Val Asp Asn Glu Glu Leu Leu Pro 185 190 195	751
AAA GGG TTA GTA GAC AGA GAA CCA CCT GAA GAG CTT GTT CAT CGA GTG Lys Gly Leu Val Asp Arg Glu Pro Pro Glu Glu Leu Val His Arg Val 200 205 210	799
AAA AGA AGA GCT GAC CCA GAT CCC ATG AAG AAC ACG TGT AAA TTA TTG Lys Arg Arg Ala Asp Pro Asp Pro Met Lys Asn Thr Cys Lys Leu Leu 215 220 225	847
GTG GTA GCA GAT CAT CGC TTC TAC AGA TAC ATG GGC AGA GGG GAA GAG Val Val Ala Asp His Arg Phe Tyr Arg Tyr Met Gly Arg Gly Glu Glu 230 235 240	895
AGT ACA ACT ACA AAT TAC TTA ATA GAG CTA ATT GAC AGA GTT GAT GAC Ser Thr Thr Thr Asn Tyr Leu Ile Glu Leu Ile Asp Arg Val Asp Asp 245 250 255 260	943
ATC TAT CGG AAC ACT TCA TGG GAT AAT CCA GGT TTT AAA GGC TAT GGA	991

Ile	Tyr	Arg	Asn	Thr	Ser	Trp	Asp	Asn	Ala	Gly	Phe	Lys	Gly	Tyr	Gly	
				265					270					275		
ATA	CAG	ATA	GAG	CAG	ATT	CGC	ATT	CTC	AAG	TCT	CCA	CAA	GAG	GTA	AAA	1039
Ile	Gln	Ile	Glu	Gln	Ile	Arg	Ile	Leu	Lys	Ser	Pro	Gln	Glu	Val	Lys	
			280					285					290			
CCT	GGT	GAA	AAG	CAC	TAC	AAC	ATG	GCA	AAA	AGT	TAC	CCA	AAT	GAA	GAA	1087
Pro	Gly	Glu	Lys	His	Tyr	Asn	Met	Ala	Lys	Ser	Tyr	Pro	Asn	Glu	Glu	
		295					300					305				
AAG	GAT	GCT	TGG	GAT	GTG	AAG	ATG	TTG	CTA	GAG	CAA	TTT	AGC	TTT	GAT	1135
Lys	Asp	Ala	Trp	Asp	Val	Lys	Met	Leu	Leu	Glu	Gln	Phe	Ser	Phe	Asp	
	310					315					320					
ATA	GCT	GAG	GAA	GCA	TCT	AAA	GTT	TGC	TTG	GCA	CAC	CTT	TTC	ACA	TAC	1183
Ile	Ala	Glu	Glu	Ala	Ser	Lys	Val	Cys	Leu	Ala	His	Leu	Phe	Thr	Tyr	
	325				330					335					340	
CAA	GAT	TTT	GAT	ATG	GGA	ACT	CTT	GGA	TTA	GCT	TAT	GTT	GGC	TCT	CCC	1231
Gln	Asp	Phe	Asp	Met	Gly	Thr	Leu	Gly	Leu	Ala	Tyr	Val	Gly	Ser	Pro	
				345					350					355		
AGA	GCA	AAC	AGC	CAT	GGA	GGT	GTT	TGT	CCA	AAG	GCT	TAT	TAT	AGC	CCA	1279
Arg	Ala	Asn	Ser	His	Gly	Gly	Val	Cys	Pro	Lys	Ala	Tyr	Tyr	Ser	Pro	
			360					365					370			
GTT	GGG	AAG	AAA	AAT	ATC	TAT	TTG	AAT	AGT	GGT	TTG	ACG	AGC	ACA	AAG	1327
Val	Gly	Lys	Lys	Asn	Ile	Tyr	Leu	Asn	Ser	Gly	Leu	Thr	Ser	Thr	Lys	
		375					380					385				
AAT	TAT	GGT	AAA	ACC	ATC	CTT	ACA	AAG	GAA	GCT	GAC	CTG	GTT	ACA	ACT	1375
Asn	Tyr	Gly	Lys	Thr	Ile	Leu	Thr	Lys	Glu	Ala	Asp	Leu	Val	Thr	Thr	
	390					395				400						
CAT	GAA	TTG	GGA	CAT	AAT	TTT	GGA	GCA	GAA	CAT	GAT	CCG	GAT	GGT	CTA	1423
His	Glu	Leu	Gly	His	Asn	Phe	Gly	Ala	Glu	His	Asp	Pro	Asp	Gly	Leu	
	405				410					415					420	
GCA	GAA	TGT	GCC	CCG	AAT	GAG	GAC	CAG	GGA	GGG	AAA	TAT	GTC	ATG	TAT	1471
Ala	Glu	Cys	Ala	Pro	Asn	Glu	Asp	Gln	Gly	Gly	Lys	Tyr	Val	Met	Tyr	
				425					430					435		
CCC	ATA	GCT	GTG	AGT	GGC	GAT	CAC	GAG	AAC	AAT	AAG	ATG	TTT	TCA	AAC	1519
Pro	Ile	Ala	Val	Ser	Gly	Asp	His	Glu	Asn	Asn	Lys	Met	Phe	Ser	Asn	
			440					445					450			
TGC	AGT	AAA	CAA	TCA	ATC	TAT	AAG	ACC	ATT	GAA	AGT	AAG	GCC	CAG	GAG	1567
Cys	Ser	Lys	Gln	Ser	Ile	Tyr	Lys	Thr	Ile	Glu	Ser	Lys	Ala	Gln	Glu	
		455					460					465				
TGT	TTT	CAA	GAA	CGC	AGC	AAT	AAA	GTT	TGT	GGG	AAC	TCG	AGG	GTG	GAT	1615
Cys	Phe	Gln	Glu	Arg	Ser	Asn	Lys	Val	Cys	Gly	Asn	Ser	Arg	Val	Asp	
	470					475					480					
GAA	GGA	GAA	GAG	TGT	GAT	CCT	GGC	ATC	ATG	TAT	CTG	AAC	AAC	GAC	ACC	1663
Glu	Gly	Glu	Glu	Cys	Asp	Pro	Gly	Ile	Met	Tyr	Leu	Asn	Asn	Asp	Thr	
	485				490					495					500	
TGC	TGC	AAC	AGC	GAC	TGC	ACG	TTG	AAG	GAA	GGT	GTC	CAG	TGC	AGT	GAC	1711
Cys	Cys	Asn	Ser	Asp	Cys	Thr	Leu	Lys	Glu	Gly	Val	Gln	Cys	Ser	Asp	
				505					510					515		
AGG	AAC	AGT	CCT	TGC	TGT	AAA	AAC	TGT	CAG	TTT	GAG	ACT	GCC	CAG	AAG	1759
Arg	Asn	Ser	Pro	Cys	Cys	Lys	Asn	Cys	Gln	Phe	Glu	Thr	Ala	Gln	Lys	
			520					525					530			

AAG TGC CAG GAG GCG ATT AAT GCT ACT TGC AAA GGC GTG TCC TAC TGC Lys Cys Gln Glu Ala Ile Asn Ala Thr Cys Lys Gly Val Ser Tyr Cys 535 540 545	1807
ACA GGT AAT AGC AGT GAG TGC CCG CCT CCA GGA AAT GCT GAA GAT GAC Thr Gly Asn Ser Ser Glu Cys Pro Pro Pro Gly Asn Ala Glu Asp Asp 550 555 560	1855
ACT GTT TGC TTG GAT CTT GGC AAG TGT AAG GAT GGG AAA TGC ATC CCT Thr Val Cys Leu Asp Leu Gly Lys Cys Lys Asp Gly Lys Cys Ile Pro 565 570 575 580	1903
TTC TGC GAG AGG GAA CAG CAG CTG GAG TCC TGT GCA TGT AAT GAA ACT Phe Cys Glu Arg Glu Gln Gln Leu Glu Ser Cys Ala Cys Asn Glu Thr 585 590 595	1951
GAC AAC TCC TGC AAG GTG TGC TGC AGG GAC CTT TCT GGC CGC TGT GTG Asp Asn Ser Cys Lys Val Cys Cys Arg Asp Leu Ser Gly Arg Cys Val 600 605 610	1999
CCC TAT GTC GAT GCT GAA CAA AAG AAC TTA TTT TTG AGG AAA GGA AAG Pro Tyr Val Asp Ala Glu Gln Lys Asn Leu Phe Leu Arg Lys Gly Lys 615 620 625	2047
CCC TGT ACA GTA GGA TTT TGT GAC ATG AAT GGC AAA TGT GAG AAA CGA Pro Cys Thr Val Gly Phe Cys Asp Met Asn Gly Lys Cys Glu Lys Arg 630 635 640	2095
GTA CAG GAT GTA ATT GAA CGA TTT TGG GAT TTC ATT GAC CAG CTG AGC Val Gln Asp Val Ile Glu Arg Phe Trp Asp Phe Ile Asp Gln Leu Ser 645 650 655 660	2143
ATC AAT ACT TTT GGA AAG TTT TTA GCA GAC AAC ATC GTT GGG TCT GTC Ile Asn Thr Phe Gly Lys Phe Leu Ala Asp Asn Ile Val Gly Ser Val 665 670 675	2191
CTG GTT TTC TCC TTG ATA TTT TGG ATT CCT TTC AGC ATT CTT GTC CAT Leu Val Phe Ser Leu Ile Phe Trp Ile Pro Phe Ser Ile Leu Val His 680 685 690	2239
TGT GTG GAT AAG AAA TTG GAT AAA CAG TAT GAA TCT CTG TCT CTG TTT Cys Val Asp Lys Lys Leu Asp Lys Gln Tyr Glu Ser Leu Ser Leu Phe 695 700 705	2287
CAC CCC AGT AAC GTC GAA ATG CTG AGC AGC ATG GAT TCT GCA TCG GTT His Pro Ser Asn Val Glu Met Leu Ser Ser Met Asp Ser Ala Ser Val 710 715 720	2335
CGC ATT ATC AAA CCC TTT CCT GCG CCC CAG ACT CCA GGC CGC CTG CAG Arg Ile Ile Lys Pro Phe Pro Ala Pro Gln Thr Pro Gly Arg Leu Gln 725 730 735 740	2383
CCT GCC CCT GTG ATC CCT TCG GCG CCA GCA GCT CCA AAA CTG GAC CAC Pro Ala Pro Val Ile Pro Ser Ala Pro Ala Ala Pro Lys Leu Asp His 745 750 755	2431
CAG AGA ATG GAC ACC ATC CAG GAA GAC CCC AGC ACA GAC TCA CAT ATG Gln Arg Met Asp Thr Ile Gln Glu Asp Pro Ser Thr Asp Ser His Met 760 765 770	2479
GAC GAG GAT GGG TTT GAG AAG GAC CCC TTC CCA AAT AGC AGC ACA GCT Asp Glu Asp Gly Phe Glu Lys Asp Pro Phe Pro Asn Ser Ser Thr Ala 775 780 785	2527
GCC AAG TCA TTT GAG GAT CTC ACG GAC CAT CCG GTC ACC AGA AGT GAA Ala Lys Ser Phe Glu Asp Leu Thr Asp His Pro Val Thr Arg Ser Glu 790 795 800	2575

AAG GCT GCC TCC TTT AAA CTG CAG CGT CAG AAT CGT GTT GAC AGC AAA 2623
 Lys Ala Ala Ser Phe Lys Leu Gln Arg Gln Asn Arg Val Asp Ser Lys
 805 810 815 820
 GAA ACA GAG TGC TAATTTAGTT CTCAGCTCTT CTGACTTAAG TGTGCAAAAT 2675
 Glu Thr Glu Cys
 ATTTTTATAG ATTTGACCTA CAATCAATCA CAGCTTATAT TTTGTGAAGA CTGGGAAGTG 2735
 ACTTAGCAGA TGCTGGTCAT GTGTTTGGAA CTTCCTGCAG GTAAACAGTT CTTGTGTGGG 2795
 TTTGGGCCCN TCTCCTTTTG GAAAAGGTAA GGGTGAAGGT GAATCTTGCT TATTNTGGGG 2855
 GTTTCAGGTT TNAGTTTTTA AAATATCTTT TGGACCTGTG GGTGNAAG CAGAAATACA 2915
 GNTGGATTGG GTTATGAGTA TTTACGT 2942

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 824 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Arg Gln Ser Leu Leu Phe Leu Thr Ser Val Val Pro Phe Val Leu
 1 5 10 15
 Ala Pro Arg Pro Pro Asp Asp Pro Gly Phe Gly Pro His Gln Arg Leu
 20 25 30
 Glu Lys Leu Asp Ser Leu Leu Ser Asp Tyr Asp Ile Leu Ser Leu Ser
 35 40 45
 Asn Ile Gln Gln His Ser Val Arg Lys Arg Asp Leu Gln Thr Ser Thr
 50 55 60
 His Val Glu Thr Leu Leu Thr Phe Ser Ala Leu Lys Arg His Phe Lys
 65 70 75 80
 Leu Tyr Leu Thr Ser Ser Thr Glu Arg Phe Ser Gln Asn Phe Lys Val
 85 90 95
 Val Val Val Asp Gly Lys Asn Glu Ser Glu Tyr Thr Val Lys Trp Gln
 100 105 110
 Asp Phe Phe Thr Gly His Val Val Gly Glu Pro Asp Ser Arg Val Leu
 115 120 125
 Ala His Ile Arg Asp Asp Asp Val Ile Ile Arg Ile Asn Thr Asp Gly
 130 135 140
 Ala Glu Tyr Asn Ile Glu Pro Leu Trp Arg Phe Val Asn Asp Thr Lys
 145 150 155 160
 Asp Lys Arg Met Leu Val Tyr Lys Ser Glu Asp Ile Lys Asn Val Ser
 165 170 175
 Arg Leu Gln Ser Pro Lys Val Cys Gly Tyr Leu Lys Val Asp Asn Glu
 180 185 190
 Glu Leu Leu Pro Lys Gly Leu Val Asp Arg Glu Pro Pro Glu Glu Leu

- 87 -

Ala Glu Asp Asp Thr Val Cys Leu Asp Leu Gly Lys Cys Lys Asp Gly
565 570 575

Lys Cys Ile Pro Phe Cys Glu Arg Glu Gln Gln Leu Glu Ser Cys Ala
580 585 590

Cys Asn Glu Thr Asp Asn Ser Cys Lys Val Cys Cys Arg Asp Leu Ser
595 600 605

Gly Arg Cys Val Pro Tyr Val Asp Ala Glu Gln Lys Asn Leu Phe Leu
610 615 620

Arg Lys Gly Lys Pro Cys Thr Val Gly Phe Cys Asp Met Asn Gly Lys
625 630 635 640

Cys Glu Lys Arg Val Gln Asp Val Ile Glu Arg Phe Trp Asp Phe Ile
645 650 655

Asp Gln Leu Ser Ile Asn Thr Phe Gly Lys Phe Leu Ala Asp Asn Ile
660 665 670

Val Gly Ser Val Leu Val Phe Ser Leu Ile Phe Trp Ile Pro Phe Ser
675 680 685

Ile Leu Val His Cys Val Asp Lys Lys Leu Asp Lys Gln Tyr Glu Ser
690 695 700

Leu Ser Leu Phe His Pro Ser Asn Val Glu Met Leu Ser Ser Met Asp
705 710 715 720

Ser Ala Ser Val Arg Ile Ile Lys Pro Phe Pro Ala Pro Gln Thr Pro
725 730 735

Gly Arg Leu Gln Pro Ala Pro Val Ile Pro Ser Ala Pro Ala Ala Pro
740 745 750

Lys Leu Asp His Gln Arg Met Asp Thr Ile Gln Glu Asp Pro Ser Thr
755 760 765

Asp Ser His Met Asp Glu Asp Gly Phe Glu Lys Asp Pro Phe Pro Asn
770 775 780

Ser Ser Thr Ala Ala Lys Ser Phe Glu Asp Leu Thr Asp His Pro Val
785 790 795 800

Thr Arg Ser Glu Lys Ala Ala Ser Phe Lys Leu Gln Arg Gln Asn Arg
805 810 815

Val Asp Ser Lys Glu Thr Glu Cys
820

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 3
- (D) OTHER INFORMATION: /mod_base= i

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTNCARGAYG TRATHGA

17

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 3
- (D) OTHER INFORMATION: /mod_base= i

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTNCARGAYG TYATHGA

17

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 3
- (D) OTHER INFORMATION: /mod_base= i

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCNACDATRT TRTCYGC

17

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCNACDATRT TRTCRGC

17

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 amino acids

- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```

Val Gln Asp Val Ile Glu Arg Phe Trp Glu Phe Ile Asp Lys Leu Ser
1           5           10           15
Ile Asn Thr Phe Gly Lys Phe Leu Ala Asp Asn Ile Val Gly Ser Val
          20           25           30
Leu Val Phe Ser Leu Met Leu Trp Ile
          35           40

```

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 89 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```

GTGCAGGACG TCATCGAGCG GTTCTGGGAG TTCATTGACA AGCTGAGCAT CAATACTTTC      60
GGGAAGTTCC TGGCAGACAA CATCGTCGG                                     89

```

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2464 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1308

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```

GAA GCT GAC CTT GTG ACA ACT CAT GAA TTG GGG CAC AAT TTT GGA GCA      48
Glu Ala Asp Leu Val Thr Thr His Glu Leu Gly His Asn Phe Gly Ala
1           5           10           15
GAA CAC GAT CCA GAT GGT TTA GCA GAA TGT GCC CCA AAC GAG GAC CAG      96
Glu His Asp Pro Asp Gly Leu Ala Glu Cys Ala Pro Asn Glu Asp Gln
          20           25           30
GGA GGA AAA TAC GTC ATG TAT CCC ATA GCC GTG AGT GGT GAT CAT GAG      144
Gly Gly Lys Tyr Val Met Tyr Pro Ile Ala Val Ser Gly Asp His Glu

```

35	40	45	192
AAC AAC AAG ATG TTT TCA AAC TGC AGT AAA CAG TCC ATC TAT AAG ACC Asn Asn Lys Met Phe Ser Asn Cys Ser Lys Gln Ser Ile Tyr Lys Thr 50 55 60			
ATT GAA AGT AAG GCC CAG GAG TGT TTT CAA GAG CGC AGC AAC AAA GTG Ile Glu Ser Lys Ala Gln Glu Cys Phe Gln Glu Arg Ser Asn Lys Val 65 70 75 80			240
TGT GGC AAC TCC AGG GTG GAT GAG GGG GAG GAG TGC GAC CCC GGC ATC Cys Gly Asn Ser Arg Val Asp Glu Gly Glu Glu Cys Asp Pro Gly Ile 85 90 95			288
ATG TAC CTG AAC AAC GAC ACC TGC TGC AAC AGC GAC TGC ACC CTG AGG Met Tyr Leu Asn Asn Asp Thr Cys Cys Asn Ser Asp Cys Thr Leu Arg 100 105 110			336
CCG GGC GTC CAG TGC AGT GAT AGG AAC AGT CCT TGC TGT AAA AAC TGT Pro Gly Val Gln Cys Ser Asp Arg Asn Ser Pro Cys Cys Lys Asn Cys 115 120 125			384
CAG TTC GAG ACG GCC CAG AAG AAG TGC CAG GAG GCT ATT AAT GCC ACT Gln Phe Glu Thr Ala Gln Lys Lys Cys Gln Glu Ala Ile Asn Ala Thr 130 135 140			432
TGC AAA GGC GTG TCT TAC TGC ACA GGT AAC AGC AGT GAG TGC CCC CCT Cys Lys Gly Val Ser Tyr Cys Thr Gly Asn Ser Ser Glu Cys Pro Pro 145 150 155 160			480
CCG GGA AAC GCC GAG GAC GAC ACG GTG TGC CTG GAC CTG GGC AGG TGC Pro Gly Asn Ala Glu Asp Asp Thr Val Cys Leu Asp Leu Gly Arg Cys 165 170 175			528
AAG GAC GGC AAG TGC GTG CCC TTC TGC GAG CGG GAG CAG CGG CTG GAG Lys Asp Gly Lys Cys Val Pro Phe Cys Glu Arg Glu Gln Arg Leu Glu 180 185 190			576
TCC TGC GCG TGT AAC GAA ACC GAC CAC TCG TGC AAG GTG TGC TGC CGG Ser Cys Ala Cys Asn Glu Thr Asp His Ser Cys Lys Val Cys Cys Arg 195 200 205			624
GCC CCC TCG GGC CGT TGG CTG CCC TAC GTG GAC GCC GAA CAG AAG AAC Ala Pro Ser Gly Arg Trp Leu Pro Tyr Val Asp Ala Glu Gln Lys Asn 210 215 220			672
TTG TTT TTG AGG AAG GGG AAG CCC TGT ACA GTA GGA TTT TGT GAC ATG Leu Phe Leu Arg Lys Gly Lys Pro Cys Thr Val Gly Phe Cys Asp Met 225 230 235 240			720
AAT GGC AAG TGT GAG AAG CGA GTG CAG GAC GTC ATC GAG CGG TTC TGG Asn Gly Lys Cys Glu Lys Arg Val Gln Asp Val Ile Glu Arg Phe Trp 245 250 255			768
GAG TTC ATT GAC AAG CTG AGC ATC AAT ACT TTC GGG AAG TTC CTG GCA Glu Phe Ile Asp Lys Leu Ser Ile Asn Thr Phe Gly Lys Phe Leu Ala 260 265 270			816
GAC AAC ATC GTG GGC TCC GTC CTG GTG TTC TCC CTG ATG CTC TGG ATC Asp Asn Ile Val Gly Ser Val Leu Val Phe Ser Leu Met Leu Trp Ile 275 280 285			864
CCC GTC AGC ATC CTC GTC CAC TGC GTG GAT AAG AAG CTG GAT AAG CAG Pro Val Ser Ile Leu Val His Cys Val Asp Lys Lys Leu Asp Lys Gln 290 295 300			912
TAC GAA TCC CTG TCT CTG CTG CAC CCC AGC AAC GTG GAG ATG CTA AGC			960

Tyr 305	Glu	Ser	Leu	Ser	Leu	Leu	His	Pro	Ser	Asn 315	Val	Glu	Met	Leu	Ser 320	
AGC Ser	ATG Met	GAT Asp	TCA Ser	GCA Ala 325	TCC Ser	GTT Val	CGC Arg	ATC Ile	ATC Ile 330	AAG Lys	CCC Pro	TTT Phe	CCT Pro	GCG Ala 335	CCC Pro	1008
CAG Gln	ACC Thr	CCA Pro	GGC Gly 340	CGC Arg	CTG Leu	CAG Gln	CCC Pro	CTG Leu 345	CAG Gln	CCC Pro	CTG Leu	CAG Gln	CCC Pro 350	GGC Gly	CCC Pro	1056
GTG Val	CTG Leu	CCC Pro 355	TCT Ser	GCG Ala	CCT Pro	TCG Ser	GTG Val 360	CCC Pro	GTG Val	GCT Ala	CCA Pro	AAA Lys 365	CTG Leu	GAC Asp	CAC His	1104
CAG Gln	CGG Arg 370	ATG Met	GAC Asp	ACC Thr	ATC Ile	CAG Gln 375	GAG Glu	GAC Asp	CCC Pro	AGC Ser	ACG Thr 380	GAC Asp	TCG Ser	CAC His	GTG Val	1152
GAC Asp 385	GAG Glu	GAC Asp	GGC Gly	TTC Phe	GAG Glu 390	AAG Lys	GAC Asp	CCT Pro	TTC Phe	CCC Pro 395	AAC Asn	AGC Ser	AGT Ser	GCC Ala	GCT Ala 400	1200
GCC Ala	AAG Lys	TCA Ser	TTT Phe 405	GAG Glu	GAT Asp	CTC Leu	ACG Thr	GAC Asp 410	CAT His	CCG Pro	GTC Val	ACG Thr	AGA Arg	AGT Ser 415	GAA Glu	1248
AAG Lys	GCC Ala	TCG Ser	TCC Ser 420	TTT Phe	AAG Lys	CTG Leu	CAG Gln	CGC Arg 425	CAG Gln	AGT Ser	CGC Arg	GTT Val	GAC Asp 430	AGC Ser	AAG Lys	1296
GAA Glu	ACG Thr	GAG Glu 435	TGC Cys	TAGTTCAGGG CTGCGTCCGG AGCTCTCGGA CTTGGCGTGC												1348
AGGATATTTT TATAGATTTG ACCTCAGATC ACTGCGGAGA TCTTGTGAAG ATTTGGGAGA																1408
AAAAAGGAAG TGA CTCTCCCA GCAGATGCTG GTCATGAGTT TGGACTTCCT CCACCTCCGT																1468
GAGACTGTGT GGGTAGCCCT TTTCCTTTGA AGAGGTAAGG CGAACCTAGC TTACTTTGAG																1528
GCCTTCAGGT TTTAGTCTTT TGATCTTTAA AATATCTTTC AACCTGTGGT GCAGAAGCAG																1588
AAACCACAGC TGGATTATGT TATGACTATT TACGTTTTTG TAAATTACCT TTATATTGAG																1648
AACAGCACTG ATTGTGGAGG TGATCATGTT TTTAAGACAC TGTAACGATC CAATGAACAC																1708
AAAGAGGCAT TTCATCAGTT TCCTGCGAGG ATAAGTGGAA CACAGAATAG ATGGATTCT																1768
TCCCTTCAGG TCAAATAAAG TCCGAGGTGC GTAATGTCAC CCAAATCTGC TTTCTGATGC																1828
AGAACCCGGT TCTACGTTCC CCCCAAAGGT GGGTTCAGGT TCGAGGTATT GCCTAGAACT																1888
TAGAGCAGAA GGGAGACCAC ACATCTGAAT TTAGGAATAA ACTGGGTTAA TGTTTCTTCC																1948
TGTTTCTAAA TGTTTCAGAAT TTCTAATTTT TAGAATTAAA ACAATCTAGC TTTATAGTAA																2008
AGATTATAAA TTATAGCCAC ATGAATTGAC AGAATCCTGC TGATTAACTG TTTAGGGCCA																2068
TACCTAGCTG GGCAGTTTTT CTGGGTTTTG TAAGTATCTT CCTGTCTCTC TACGGGTGCC																2128
ACGCTCCTAA AAAACCCACC ATCCACAAAA CCTCAACCCT TCAGAACAGG AGGACGGTCG																2188
CCTCTTCGCA AACACTAGTG GCCCAGACGG TCGGTGCGGT TGGGCGCGCT TGCCAGGCGA																2248
ACAGGGTGCG AGGCGCTGGC CGGGGGTCTG TCCTCAGACC ATTGGTCCCT AGACCCTAGA																2308

CCTTCTGGCT CGCGTGCTTC GGCACCGTCT GCGCGCCCTG CGCGAGGGAT AAAAACCATC 2368
 CGGCTGAGTT CTGGTTTCAA AAGCTTTTTC CCCTGCTAAG GACTGCAGTG TGCTCACCAG 2428
 CCAGCACCCC TTAACAGGTA ACTTGTAAGC TTCGAG 2464

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 436 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Glu Ala Asp Leu Val Thr Thr His Glu Leu Gly His Asn Phe Gly Ala
 1 5 10 15
 Glu His Asp Pro Asp Gly Leu Ala Glu Cys Ala Pro Asn Glu Asp Gln
 20 25 30
 Gly Gly Lys Tyr Val Met Tyr Pro Ile Ala Val Ser Gly Asp His Glu
 35 40 45
 Asn Asn Lys Met Phe Ser Asn Cys Ser Lys Gln Ser Ile Tyr Lys Thr
 50 55 60
 Ile Glu Ser Lys Ala Gln Glu Cys Phe Gln Glu Arg Ser Asn Lys Val
 65 70 75 80
 Cys Gly Asn Ser Arg Val Asp Glu Gly Glu Glu Cys Asp Pro Gly Ile
 85 90 95
 Met Tyr Leu Asn Asn Asp Thr Cys Cys Asn Ser Asp Cys Thr Leu Arg
 100 105 110
 Pro Gly Val Gln Cys Ser Asp Arg Asn Ser Pro Cys Cys Lys Asn Cys
 115 120 125
 Gln Phe Glu Thr Ala Gln Lys Lys Cys Gln Glu Ala Ile Asn Ala Thr
 130 135 140
 Cys Lys Gly Val Ser Tyr Cys Thr Gly Asn Ser Ser Glu Cys Pro Pro
 145 150 155 160
 Pro Gly Asn Ala Glu Asp Asp Thr Val Cys Leu Asp Leu Gly Arg Cys
 165 170 175
 Lys Asp Gly Lys Cys Val Pro Phe Cys Glu Arg Glu Gln Arg Leu Glu
 180 185 190
 Ser Cys Ala Cys Asn Glu Thr Asp His Ser Cys Lys Val Cys Cys Arg
 195 200 205
 Ala Pro Ser Gly Arg Trp Leu Pro Tyr Val Asp Ala Glu Gln Lys Asn
 210 215 220
 Leu Phe Leu Arg Lys Gly Lys Pro Cys Thr Val Gly Phe Cys Asp Met
 225 230 235 240
 Asn Gly Lys Cys Glu Lys Arg Val Gln Asp Val Ile Glu Arg Phe Trp
 245 250 255
 Glu Phe Ile Asp Lys Leu Ser Ile Asn Thr Phe Gly Lys Phe Leu Ala

260	265	270
Asp Asn Ile Val Gly Ser Val Leu Val Phe Ser Leu Met Leu Trp Ile		
275	280	285
Pro Val Ser Ile Leu Val His Cys Val Asp Lys Lys Leu Asp Lys Gln		
290	295	300
Tyr Glu Ser Leu Ser Leu Leu His Pro Ser Asn Val Glu Met Leu Ser		
305	310	315
Ser Met Asp Ser Ala Ser Val Arg Ile Ile Lys Pro Phe Pro Ala Pro		
325	330	335
Gln Thr Pro Gly Arg Leu Gln Pro Leu Gln Pro Leu Gln Pro Gly Pro		
340	345	350
Val Leu Pro Ser Ala Pro Ser Val Pro Val Ala Pro Lys Leu Asp His		
355	360	365
Gln Arg Met Asp Thr Ile Gln Glu Asp Pro Ser Thr Asp Ser His Val		
370	375	380
Asp Glu Asp Gly Phe Glu Lys Asp Pro Phe Pro Asn Ser Ser Ala Ala		
385	390	395
Ala Lys Ser Phe Glu Asp Leu Thr Asp His Pro Val Thr Arg Ser Glu		
405	410	415
Lys Ala Ser Ser Phe Lys Leu Gln Arg Gln Ser Arg Val Asp Ser Lys		
420	425	430
Glu Thr Glu Cys		
435		

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Val Gln Asp Val Ile Glu
1 5

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ala Asp Asn Ile Val Gly
1 5

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 55 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCGGTTCTGG GAGTTCATTG ACAAGCTGAG CATCAATACT TTCGGGAAGT TCCTG

55

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 13 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: unknown
 - (ii) MOLECULE TYPE: peptide
 - (ix) FEATURE:
 - (A) NAME/KEY: Modified-site
 - (B) LOCATION: 1
 - (D) OTHER INFORMATION: /product= "OTHER"
- /note= "X = Dinitrophenylalanine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Xaa Ser Pro Leu Ala Gln Ala Val Arg Ser Ser Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: unknown
- (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Val Gln Asp Val Ile Glu Arg Phe Trp Glu Phe Ile Asp Lys Leu Ser
1 5 10 15
Ile Asn Thr Phe Gly Lys Phe Leu Ala Asp Asn Ile Val Gly
20 25 30

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "A biotin molecule is N-terminally linked to leucine"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 9
- (D) OTHER INFORMATION: /note= "Dinitrophenylalanine-NH2 is C-terminally linked to lysine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Leu Ala Gln Ala Val Arg Ser Ser Lys
1 5

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "A fluorophore such as NBD is N-terminally linked to serine"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 10
- (D) OTHER INFORMATION: /note= "DMC (dimethyl coumarin) is linked as a side group to Lys"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ser Pro Leu Ala Gln Ala Val Arg Ser Lys Ser Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "A biotin molecule is
N-terminally linked to leucine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 9
(D) OTHER INFORMATION: /note= "Dinitrophenylalanine-NH2 is
C-terminally linked to lysine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Leu Ala Gln Ala Val Arg Ser Ser Lys
1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "A biotin molecule is
N-terminally linked to leucine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 5
(D) OTHER INFORMATION: /note= "X = phenylglycine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 9
(D) OTHER INFORMATION: /note= "Dinitrophenylalanine-NH2 is
C-terminally linked to lysine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Leu Ala Gln Ala Xaa Ala Ser Ser Lys
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "A biotin molecule is
N-terminally linked to leucine"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 5
- (D) OTHER INFORMATION: /note= "X = Homophenylalanine"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 9
- (D) OTHER INFORMATION: /note= "Dinitrophenylalanine-NH2 is C-terminally linked to lysine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Leu Ala Gln Ala Xaa Ala Ser Ser Lys
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "A biotin molecule is N-terminally linked to leucine"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 5
- (D) OTHER INFORMATION: /note= "X = Phenylglycine"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 9
- (D) OTHER INFORMATION: /note= "Dinitrophenylalanine-NH2 is C-terminally bound to lysine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Leu Ala Gln Ala Xaa Arg Ser Ser Lys
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "A biotin molecule is N-terminally linked to leucine"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 5

(D) OTHER INFORMATION: /note= "X = 3-(3-pyridyl) Alanine"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 8

(D) OTHER INFORMATION: /note= "Dinitrophenylalanine-NH2 is C-terminally linked to lysine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Leu Ala Gln Ala Xaa His Ser Ser Lys
1 5

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 1

(D) OTHER INFORMATION: /note= "A biotin molecule is N-terminally linked to leucine"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 5

(D) OTHER INFORMATION: /product= "X = Nle"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 9

(D) OTHER INFORMATION: /note= "Dinitrophenylalanine-NH2 is C-terminally bound to lysine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Leu Ala Gln Ala Xaa Arg Ser Ser Lys
1 5

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 1

(D) OTHER INFORMATION: /note= "A biotin molecule is N-terminally linked to leucine"

(ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 5
 (D) OTHER INFORMATION: /product= "X = Nle"

(ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 9
 (D) OTHER INFORMATION: /note= "Dinitrophenylalanine-NH2 is
C-terminally bound to lysine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Leu Ala Gln Ala Xaa Ala Ser Ser Lys
1 5

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 1
 (D) OTHER INFORMATION: /note= "Dinitrophenylalanine is
N-terminally bound to serine"

(ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 12
 (D) OTHER INFORMATION: /note= "NH2 is C-terminally bound
to arginine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Ser Pro Leu Ala Gln Ala Val Arg Ser Ser Ser Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 11 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 1
 (D) OTHER INFORMATION: /note= "Dinitrophenylalanine is
N-terminally bound to serine"

(ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 11
 (D) OTHER INFORMATION: /note= "NH2 is C-terminally bound

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Ser Pro Leu Ala Gln Ala Val Arg Ser Ser Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Dinitrophenylalanine is
N-terminally bound to serine"

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: 10
(D) OTHER INFORMATION: /note= "NH2 is C-terminally bound
to serine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Ser Pro Leu Ala Gln Ala Val Arg Ser Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: 9
(D) OTHER INFORMATION: /note= "NH2 is C-terminally bound
to serine"

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Dinitrophenylalanine is
N-terminally bound to serine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Ser Pro Leu Ala Gln Ala Val Arg Ser
1 5

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 11 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 1
 (D) OTHER INFORMATION: /note= "Dinitrophenylalanine is
N-terminally bound to proline"

(ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 11
 (D) OTHER INFORMATION: /note= "NH2 is C-terminally bound
to arginine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Pro	Leu	Ala	Gln	Ala	Val	Arg	Ser	Ser	Ser	Arg
1				5					10	

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 10
 (D) OTHER INFORMATION: /note= "NH2 is C-terminally bound
to arginine"

(ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 1
 (D) OTHER INFORMATION: /note= "Dinitrophenylalanine is
N-terminally bound to serine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Leu	Ala	Gln	Ala	Val	Arg	Ser	Ser	Ser	Arg
1				5					10

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 9 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 1
 (D) OTHER INFORMATION: /note= "Dinitrophenylalanine is
N-terminally linked to alanine"

(ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 9
 (D) OTHER INFORMATION: /note= "NH2 is C-terminally bound
to arginine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Ala Gln Ala Val Arg Ser Ser Ser Arg
1 5

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 22 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CCTAGAGTCA GGCTCACCAA CC

22

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 48 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CACGCGTCGA CTAGTTACCA TCCACCACCA CGACCTTGAA ATTTTGTG

48

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 48 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

 (A) NAME/KEY: modified_base
 (B) LOCATION: 36
 (D) OTHER INFORMATION: /mod_base= i

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 37
 (D) OTHER INFORMATION: /mod_base= i

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 41
 (D) OTHER INFORMATION: /mod_base= i

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 42
 (D) OTHER INFORMATION: /mod_base= i

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 46
 (D) OTHER INFORMATION: /mod_base= i

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 47
 (D) OTHER INFORMATION: /mod_base= i

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CUACUACUAC UAGGCCACGC GTCGACTAGT ACGGGNNGG NNGGGNNG

48

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 35 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CGGGATCCAT GAGGCAGTCT CTCCTATTCC TGACC

35

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 53 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CAGGAAGTTG CGGCCGCTGA CCAGCATCTG CTAAGTCACT TCCAGTCTT CAC

53

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 54 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GCGCGCGCGC CATATGTTAG TTTATAAATC TGAAGATATC AAGAATGTTT CACG

54

(2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 51 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CGCGCGCGCG GGATCCCTAT CGTTCAATTA CATCCTGTAC TCGTTTCTCA C

51

(2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 518 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

AAACTGGCTG AACCGGCCTT TTGGTAACGC CACCTTCACT TCTGGGGGCG TCGAACCTGG	60
CGGTAGAATC TTCCCAGTAG GCGGCGCGGG AGGGAAGA GATTGAGGG GCTAGGCCGG	120
GCGGATCCCG TCCTCCCCCG ATGTGAGCAG TTTTCCGAAA CCCCCTCAGG CGAAGGCTGC	180
CCAGAGAGGT GGAGTCGGTA GCGGGGCCGG GAACATGAGG CAGTCTCTCC TATTCCTGAC	240
CAGCGTGGTT CCTTTCGTGC TGGCGCCGCG ACCTCCGGAT GACCCGGGCT TCGGCCCCCA	300
CCAGAGACTC GAGGAGCTTG ATTCTTTGCT CTCAGACTAC GATATTCTCT CTTTATCTAA	360
TATCCAGCAG CATTCGGTAA GAAAAAGAGA TCTACAGACT TCAACACATG TAGAAACACT	420
ACTAACTTTT TCAGCTTTGA AAAGGCATTT TAAATTATAC CTGACATCAA GTACTGAACG	480
TTTTTCACAA AATTTCAAGG TCGTGGTGGT GGATGGTA	518

WHAT IS CLAIMED IS:

1. An isolated DNA sequence encoding a biologically active TNF α -convertase.
- 5 2. The isolated DNA sequence of claim 1, wherein the TNF α -convertase is from a mammal.
3. The isolated DNA sequence of claim 2, wherein the TNF α -convertase is from a human.
- 10 4. The isolated DNA of claim 3, having the nucleotide sequence shown in FIGURE 1 (SEQ ID NO 1).
5. An isolated DNA, which is capable of
15 hybridizing under highly stringent or moderately stringent conditions to a DNA sequence that is complementary to the DNA sequence of claim 1 (SEQ ID NO 1).
6. A recombinant DNA expression vector comprising
20 the DNA of claim 1.
7. The recombinant DNA expression vector of claim 6, in which the DNA is operatively associated with a regulatory sequence that controls expression of the DNA in a
25 host.
8. The recombinant DNA expression vector of claim 7, which further comprises a DNA sequence encoding a selectable marker or reporter gene product.
- 30 9. A host cell containing the recombinant DNA expression vector of claim 6.
10. The host cell of claim 9 that expresses a
35 biologically active TNF α -convertase.

11. A substantially pure mammalian TNF α -convertase.

12. The substantially pure TNF α -convertase of claim 11, wherein the mammal is a human.

13. The substantially pure TNF α -convertase of claim 11, wherein the mammal is a pig.

10 14. A substantially pure TNF α -convertase having the amino acid sequence shown in FIGURE 1 (SEQ ID NO 2).

15 15. A fusion protein comprising a TNF α -convertase linked to a heterologous protein or peptide sequence.

16. A method for isolating a recombinant TNF α -convertase, comprising culturing the host cell of claim 9 under conditions conducive to the production of a biologically active TNF α -convertase, and recovering
20 TNF α -convertase from the cell culture.

17. A recombinant TNF α -convertase produced by the method of claim 16.

25 18. A method for isolating a compound capable of binding to TNF α -convertase, comprising:

- (a) immobilizing TNF α -convertase or a portion thereof by conjugation to a solid phase matrix;
- 30 (b) contacting the TNF α -convertase-solid phase matrix conjugate of (a) with a material comprising a compound under conditions that permit the compound to bind to the immobilized TNF α -convertase;
- 35 (c) removing unbound material from the solid phase matrix;

- (d) detecting the presence of compound bound to the TNF α -convertase;
- (e) eluting the bound compound from the TNF α -convertase; and
- 5 (f) collecting the eluted compound.

19. A compound capable of binding to TNF α -convertase isolated by the method of claim 18.

- 10 20. The compound of claim 19, which inhibits a biological activity of TNF α -convertase.

21. A method for producing TNF α -convertase comprising:

- 15 (a) transfecting cells with a recombinant DNA expression vector comprising the DNA sequence of claim 1 (SEQ ID NO 1);
- (b) growing the cells of step (a) in culture medium under conditions conducive to
- 20 expression of the DNA sequence and production of TNF α -convertase; and
- (c) isolating the TNF α -convertase.

22. The method of claim 21, wherein the

25 TNF α -convertase is isolated by:

- (a) contacting the TNF α -convertase with a TNF α -convertase inhibitor that further comprises a biotin moiety under conditions conducive to binding of the TNF α -convertase
- 30 to the inhibitor-biotin moiety so as to form a TNF α -convertase-inhibitor-biotin conjugate;
- (b) contacting the TNF α -convertase-inhibitor-biotin conjugate of (a) with streptavidin bound to a solid phase matrix under
- 35 conditions conducive to binding of the TNF α -convertase-inhibitor-biotin conjugate to the streptavidin;

- (c) removing unbound material; and
(d) eluting the TNF α -convertase-inhibitor-biotin conjugate from the streptavidin or eluting the TNF α -convertase from the inhibitor-biotin conjugate.

23. A method of screening for compounds that modulate the level of TNF α in a mammalian subject, comprising testing compounds for their ability to modulate a biological activity of TNF α -convertase.

24. The method of claim 23, wherein the biological activity of TNF α -convertase is selected from the group consisting of detectable binding to TNF α precursor, or to TNF α , or to a synthetic substrate, conversion of TNF α precursor to mature TNF α , and cleavage of a synthetic substrate.

25. A method of treating a disease or condition characterized by an elevated level of TNF α in the serum or tissues of a mammalian subject, comprising administering an effective amount of a compound that modulates the biological activity of TNF α -convertase to a mammalian subject in need of said treatment.

26. The method of claim 25, wherein the disease or condition is selected from the group consisting of systemic inflammatory response syndrome, reperfusion injury, cardiovascular disease, infectious disease, obstetrical disorders, gynecological disorders, inflammatory disease, autoimmunity, allergic disease, atopic disease, malignancy, transplant complication

27. The method of claim 25, wherein the disease or conditions is selected from the group consisting of septic shock, cachexia, AIDS, graft-versus-host disease, cerebral malaria, Crohn's disease, diabetes, inflammatory

bowel disease, osteoporosis, restenosis, psoriasis, infarction, and rheumatoid arthritis, macular degeneration, osteoarthritis, and multiple sclerosis.

5 28. The method of claim 25, wherein the disease or condition is infarction due to an ischemic event.

 29. A pharmaceutical composition, comprising a compound that inhibits TNF α -convertase and a
10 pharmaceutically acceptable carrier.

 30. An oligonucleotide which encodes an antisense sequence complementary to a portion of a TNF α -convertase coding sequence, and which inhibits transcription or
15 translation of the TNF α -convertase coding sequence in a cell.

 31. A complex comprising TNF α -convertase having the amino acid sequence shown in Figure 1 (SEQ ID NO 1) or a
20 portion thereof and a therapeutic agent capable of modulating the activity of the TNF α -convertase.

 32. An inhibitor of TNF α -convertase, having the chemical structure shown in Figure 8.

25

 33. The inhibitor of claim 32, wherein R comprises a biotin moiety.

 34. The inhibitor of claim 33, wherein R is
30 selected from the group consisting of $-(CH_2)_n$ -Biotin, where $n = 0-10$; $-(CH_2)_n$ -Imino Biotin, where $n = 0-10$; $-(CH_2)_n$ -S-S- $(CH_2)_n$ -Biotin, where $n = 0-10$; and $-(CH_2)_n$ -S-S- $(CH_2)_n$ -Imino Biotin, where $n = 0-10$.

35 35. A method of isolating TNF α -convertase comprising contacting a preparation comprising TNF α -convertase with the inhibitor of claim 32 under conditions

conducive to the binding of the TNF α -convertase to the inhibitor to form a TNF α -convertase-complex and isolating the TNF α -convertase-complex.

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CGAGCCTGGC GGTAGAAATCT TCCAGTAGG CGGCGCGGA GGAAAGAG GATTGAGGGG 60
 CTAGGCCGGG CGGATCCCGT CCTCCCCGA TGTGAGCAGT TTTCCGAAAC CCCGTCAGGC 120
 GAAGGCTGCC CAGAGAGGTG GAGTCGGTAG CGGGGCCGGG AAC ATG AGG CAG TCT 175
 Met Arg Gln Ser 1
 CTC CTA TTC CTG ACC AGC GTG GTT CCT TTC GTG CTG GCG CCG CGA CCT 223
 Leu Leu Phe Leu Thr Ser Val Val Pro Phe Val Leu Ala Pro Arg Pro 20
 5 10 15 20
 CCG GAT GAC CCG GGC TTC GGC CCC CAC CAG AGA CTC GAG AAG CTT GAT 271
 Pro Asp Asp Pro Gly Phe Phe Gly Pro His Gln Arg Leu Glu Lys Leu Asp 35
 25 30 35
 TCT TTG CTC TCA GAC TAC GAT ATT CTC TCT TTA TCT AAT ATC CAG CAG 319
 Ser Leu Leu Ser Asp Tyr Asp Ile Leu Ser Leu Ser Asn Ile Gln Gln 50
 40 45 50
 CAT TCG GTA AGA AAA AGA GAT CTA CAG ACT TCA ACA CAT GTA GAA ACA 367
 His Ser Val Arg Lys Arg Asp Leu Gln Thr Ser Thr His Val Glu Thr 65
 55 60 65
 CTA CTA ACT TTT TCA GCT TTG AAA AGG CAT TTT AAA TTA TAC CTG ACA 415
 Leu Leu Thr Phe Ser Ala Leu Lys Arg His Phe Lys Leu Tyr Leu Thr 80
 70 75 80
 TCA AGT ACT GAA CGT TTT TCA CAA AAT TTC AAG GTC GTG GTG GAT 463
 Ser Ser Thr Glu Arg Phe Ser Gln Asn Phe Lys Val Val Val Val Asp 100
 85 90 95 100

FIG. 1(I)

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511 GGT AAA AAC GAA AGC GAG TAC ACT GTA AAA TGG CAG GAC TTC TTC ACT
 Gly Lys Asn Glu Ser Glu Tyr Thr Val Lys Trp Gln Asp Phe Thr
 105 110 115

 559 GGA CAC GTG GTT GGT GAG CCT GAC TCT AGG GTT CTA GCC CAC ATA AGA
 Gly His Val Val Gly Glu Pro Asp Ser Arg Val Leu Ala His Ile Arg
 120 125 130

 607 GAT GAT GAT GTT ATA ATC AGA ATC AAC ACA GAT GGG GCC GAA TAT AAC
 Asp Asp Asp Val Ile Ile Arg Ile Asn Thr Asp Gly Ala Glu Tyr Asn
 135 140 145

 655 ATA GAG CCA CTT TGG AGA TTT GTT AAT GAT ACC AAA GAC AAA AGA ATG
 Ile Glu Pro Leu Trp Arg Phe Val Asn Asp Thr Lys Asp Lys Arg Met
 150 155 160

 703 TTA GTT TAT AAA TCT GAA GAT ATC AAG AAT GTT TCA CGT TTG CAG TCT
 Leu Val Tyr Lys Ser Glu Asp Ile Lys Asn Val Ser Arg Leu Gln Ser
 165 170 175 180

 751 CCA AAA GTG TGT GGT TAT TTA AAA GTG GAT AAT GAA GAG TTG CTC CCA
 Pro Lys Val Cys Gly Tyr Leu Lys Val Asp Asn Glu Glu Leu Pro
 185 190 195

 799 AAA GGG TTA GTA GAC AGA GAA CCA CCT GAA GAG CTT GTT CAT CGA GTG
 Lys Gly Leu Val Asp Arg Glu Pro Pro Glu Glu Leu Val His Arg Val
 200 205 210

 847 AAA AGA AGA GCT GAC CCA GAT CCC ATG AAG AAC ACG TGT AAA TTA TTG
 Lys Arg Arg Ala Asp Pro Asp Pro Met Lys Asn Thr Cys Lys Leu Leu
 215 220 225

FIG. 1(II)

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GTG GTA GCA GAT CAT CGC TTC TAC AGA TAC ATG GGC AGA GGC GAA GAG Val Val Ala Asp His Arg Phe Tyr Arg Tyr Met Gly Arg Gly Glu Glu 230 235 240	895
AGT ACA ACT ACA AAT TAC TTA ATA GAG CTA ATT GAC AGA GTT GAT GAC Ser Thr Thr Asn Tyr Leu Ile Glu Leu Ile Asp Arg Val Asp Asp 245 250 255 260	943
ATC TAT CGG AAC ACT TCA TGG GAT AAT GCA GGT TTT AAA GGC TAT GGA Ile Tyr Arg Asn Thr Ser Trp Asp Asn Ala Gly Phe Lys Gly Tyr Gly 265 270 275	991
ATA CAG ATA GAG CAG ATT CGC ATT CTC AAG TCT CCA CAA GAG GTA AAA Ile Gln Ile Glu Gln Ile Arg Ile Leu Lys Ser Pro Gln Glu Val Lys 280 285 290	1039
CCT GGT GAA AAG CAC TAC AAC ATG GCA AAA AGT TAC CCA AAT GAA GAA Pro Gly Glu Lys His Tyr Asn Met Ala Lys Ser Tyr Pro Asn Glu Glu 295 300 305	1087
AAG GAT GCT TGG GAT GTG AAG ATG TTG CTA GAG CAA TTT AGC TTT GAT Lys Asp Ala Trp Asp Val Lys Met Leu Leu Glu Gln Phe Ser Phe Asp 310 315 320	1135
ATA GCT GAG GAA GCA TCT AAA GTT TGC TTG GCA CAC CTT TTC ACA TAC Ile Ala Glu Glu Ala Ser Lys Val Cys Leu Ala His Leu Phe Thr Tyr 325 330 335 340	1183
CAA GAT TTT GAT ATG GGA ACT CTT GGA TTA GCT TAT GTT GGC TCT CCC Gln Asp Phe Asp Met Gly Thr Leu Gly Leu Ala Tyr Val Gly Ser Pro 345 350 355	1231

FIG. 1(III)

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AGA GCA AAC AGC CAT GGA GGT GTT TGT CCA AAG GCT TAT TAT AGC CCA Arg Ala Asn Ser His Gly Gly Val Cys Pro Lys Ala Tyr Tyr Ser Pro 360 365 370	1279
GTT GGG AAG AAA AAT ATC TAT TTG AAT AGT GGT TTG ACG AGC ACA AAG Val Gly Lys Lys Asn Ile Tyr Leu Asn Ser Gly Leu Thr Ser Thr Lys 375 380 385	1327
AAT TAT GGT AAA ACC ATC CTT ACA AAG GAA GCT GAC CTG GTT ACA ACT Asn Tyr Gly Lys Thr Ile Leu Thr Lys Lys Glu Ala Asp Leu Val Thr Thr 390 395 400	1375
CAT GAA TTG GGA CAT AAT TTT GGA GCA GAA CAT GAT CCG GAT GGT CTA His Glu Leu Gly His Asn Phe Gly Ala Glu His Asp Pro Asp Gly Leu 405 410 415 420	1423
GCA GAA TGT GCC CCG AAT GAG GAC CAG GGA GGG AAA TAT GTC ATG TAT Ala Glu Cys Ala Pro Asn Glu Asp Gln Gly Gly Lys Tyr Val Met Tyr 425 430 435	1471
CCC ATA GCT GTG AGT GGC GAT CAC GAG AAC AAT AAG ATG TTT TCA AAC Pro Ile Ala Val Ser Gly Asp His Glu Asn Asn Lys Met Phe Ser Asn 440 445 450	1519
TGC AGT AAA CAA TCA ATC TAT AAG ACC ATT GAA AGT AAG GCC CAG GAG Cys Ser Lys Gln Ser Ile Tyr Lys Thr Ile Glu Ser Lys Ala Gln Glu 455 460 465	1567
TGT TTT CAA GAA CGC AGC AAT AAA GTT TGT GGG AAC TCG AGG GTG GAT Cys Phe Gln Glu Arg Ser Asn Lys Val Cys Gly Asn Ser Arg Val Asp 470 475 480	1615

FIG. 1(IV)

5 / 20

GAA GGA GAA GAG TGT GAT CCT GGC ATC ATG TAT CTG AAC AAC GAC ACC Glu Gly Glu Glu Cys Asp Pro Gly Ile Met Tyr Leu Asn Asn Asp Thr 485 490 500	1663
TGC TGC AAC AGC GAC TGC ACG TTG AAG GAA GGT GTC CAG TGC AGT GAC Cys Cys Asn Ser Asp Cys Thr Leu Lys Glu Gly Val Gln Cys Ser Asp 505 510 515	1711
AGG AAC AGT CCT TGC TGT AAA AAC TGT CAG TTT GAG ACT GCC CAG AAG Arg Asn Ser Pro Cys Cys Lys Asn Cys Gln Phe Glu Thr Ala Gln Lys 520 525 530	1759
AAG TGC CAG GAG GCG ATT AAT GCT ACT TGC AAA GGC GTG TCC TAC TGC Lys Cys Gln Glu Ala Ile Asn Ala Thr Cys Lys Gly Val Ser Tyr Cys 535 540 545	1807
ACA GGT AAT AGC AGT GAG TGC CCG CCT CCA GGA AAT GCT GAA GAT GAC Thr Gly Asn Ser Ser Glu Cys Pro Pro Gly Asn Ala Glu Asp Asp 550 555 560	1855
ACT GTT TGC TTG GAT CTT GGC AAG TGT AAG GAT GGG AAA TGC ATC CCT Thr Val Cys Cys Leu Asp Leu Gly Lys Cys Lys Asp Gly Lys Cys Ile Pro 565 570 575 580	1903
TTC TGC GAG AGG GAA CAG CAG CTG GAG TCC TGT GCA TGT AAT GAA ACT Phe Cys Glu Arg Arg Glu Gln Leu Glu Ser Cys Ala Cys Asn Glu Thr 585 590 595	1951
GAC AAC TCC TGC AAG GTG TGC TGC AGG GAC CTT TCT GGC CGC TGT GTG Asp Asn Ser Cys Lys Val Cys Cys Arg Asp Leu Ser Gly Arg Cys Val 600 605 610	1999

FIG. 1(V)

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CCC TAT GTC GAT GCT GAA CAA AAG AAC TTA TTT TTG AGG AAA GGA AAG Pro Tyr Val Asp Ala Glu Gln Lys Asn Leu Phe Leu Arg Lys Gly Lys 615 620 625	2047
CCC TGT ACA GTA GGA TTT TGT GAC ATG AAT GGC AAA TGT GAG AAA CGA Pro Cys Thr Val Gly Phe Cys Asp Met Asn Gly Lys Cys Glu Lys Arg 630 635 640	2095
GTA CAG GAT GTA ATT GAA CGA TTT TGG GAT TTC ATT GAC CAG CTG AGC Val Gln Asp Val Ile Glu Arg Phe Trp Asp Phe Ile Asp Gln Leu Ser 645 650 655 660	2143
ATC AAT ACT TTT GGA AAG TTT TTA GCA GAC AAC ATC GTT GGG TCT GTC Ile Asn Thr Phe Gly Lys Phe Leu Ala Asp Asn Ile Val Gly Ser Val 665 670 675	2191
CTG GTT TTC TCC TTG ATA TTT TGG ATT CCT TTC AGC ATT CTT GTC CAT Leu Val Phe Ser Leu Ile Phe Trp Ile Pro Phe Ser Ile Leu Val His 680 685 690	2239
TGT GTG GAT AAG AAA TTG GAT AAA CAG TAT GAA TCT CTG TCT CTG TTT Cys Val Asp Lys Lys Leu Asp Lys Gln Tyr Glu Ser Leu Ser Leu Phe 695 700 705	2287
CAC CCC AGT AAC GTC GAA ATG CTG AGC AGC ATG GAT TCT GCA TCG GTT His Pro Ser Asn Val Glu Met Leu Ser Ser Met Asp Ser Ala Ser Val 710 715 720	2335
CGC ATT ATC AAA CCC TTT CCT GCG CCC CAG ACT CCA GGC CGC CTG CAG Arg Ile Ile Lys Pro Phe Pro Ala Pro Gln Thr Pro Gly Arg Leu Gln 725 730 735 740	2383

FIG. 1(VI)

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CCT GCC CCT GTG ATC CCT TCG GCG CCA GCA GCT CCA AAA CTG GAC CAC 2431
 Pro Ala Pro Val Ile Pro Ser Ala Pro Ala Ala Pro Lys Leu Asp His 755
 745
 CAG AGA ATG GAC ACC ATC CAG GAA GAC CCC AGC ACA GAC TCA CAT ATG 2479
 Gln Arg Met Asp Thr Ile Gln Glu Asp Pro Ser Thr Asp Ser His Met 770
 760
 GAC GAG GAT GGG TTT GAG AAG GAC CCC TTC CCA AAT AGC AGC ACA GCT 2527
 Asp Glu Asp Gly Phe Glu Lys Asp Pro Phe Pro Asn Ser Ser Thr Ala 785
 775
 GCC AAG TCA TTT GAG GAT CTC ACG GAC CAT CCG GTC ACC AGA AGT GAA 2575
 Ala Lys Ser Phe Glu Asp Leu Thr Asp His Pro Val Thr Arg Ser Glu 800
 790
 AAG GCT GCC TCC TTT AAA CTG CAG CGT CAG AAT CGT GTT GAC AGC AAA 2623
 Lys Ala Ala Ser Phe Lys Leu Gln Arg Gln Asn Arg Val Asp Ser Lys 810
 805
 GAA ACA GAG TGC TAA TTTAGTTCTC AGCTCTTCTG ACTTAAAGTGT GCAAAATATT 2678
 Glu Thr Glu Cys ***
 TTTATAGATT TGACCTACAA TCAATCACAG CTTATATTTT GTGAAGACTG GGAAGTGACT 2738
 TAGCAGATGC TGGTCATGTG TTTGGAACCT CCTGCAGGTA AACAGTTCTT GTGTGGGTTT 2798
 GGGCCCNCTC CCTTTTGGAA AAGGTAAGGG TGAAGGTGAA TCTTGCTTAT TNTGGGGTT 2858
 TCAGGTTTNA GTTTTTTAAA TATCTTTTGG ACCTGTGGGT GNAAAAGCAG AAATACAGNT 2918
 GGATTGGGTT ATGAGTATTT ACGT 2942

FIG. 1(VII)

GLYCEROL DENSITY GRADIENT:

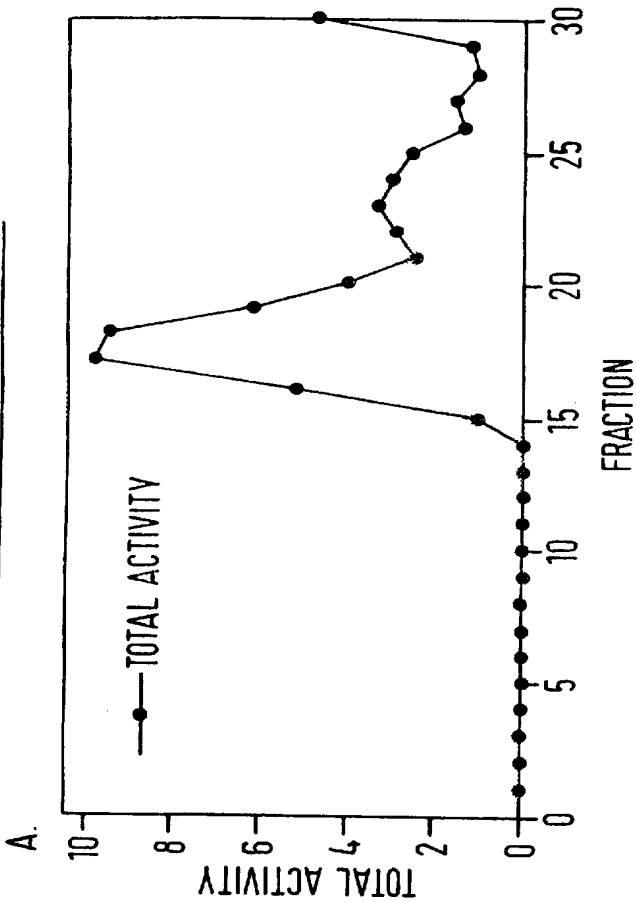
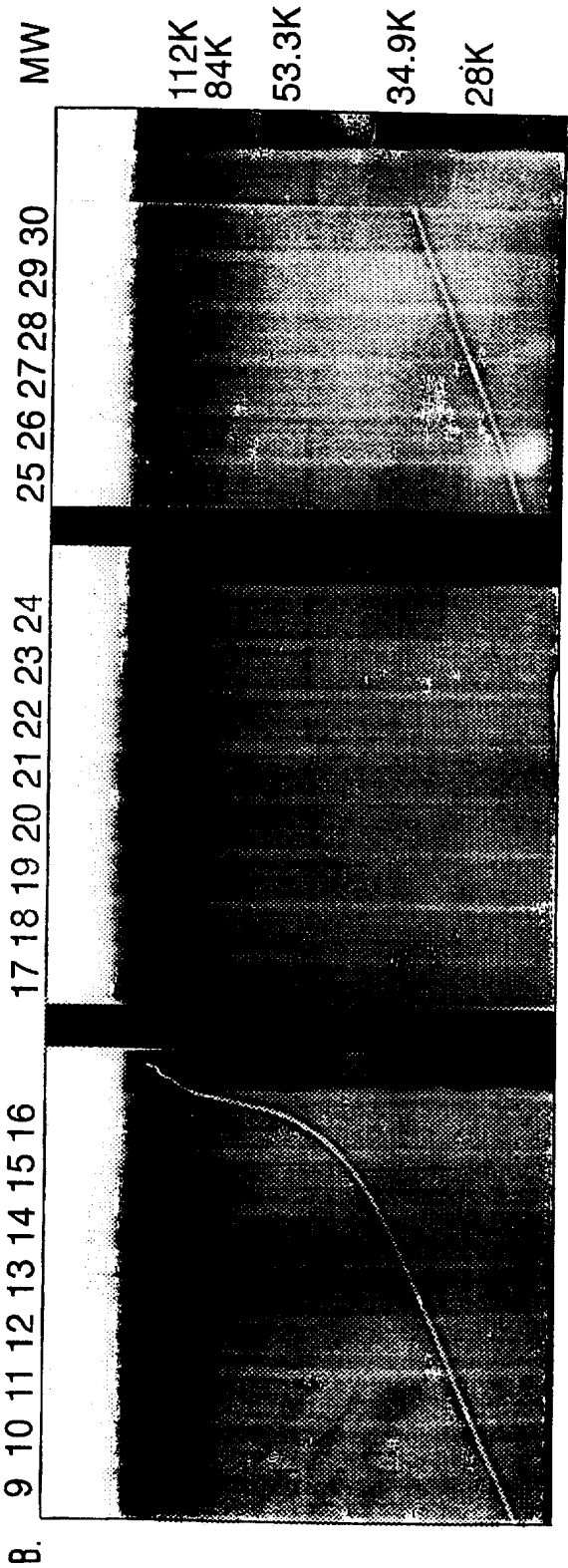


FIG. 2



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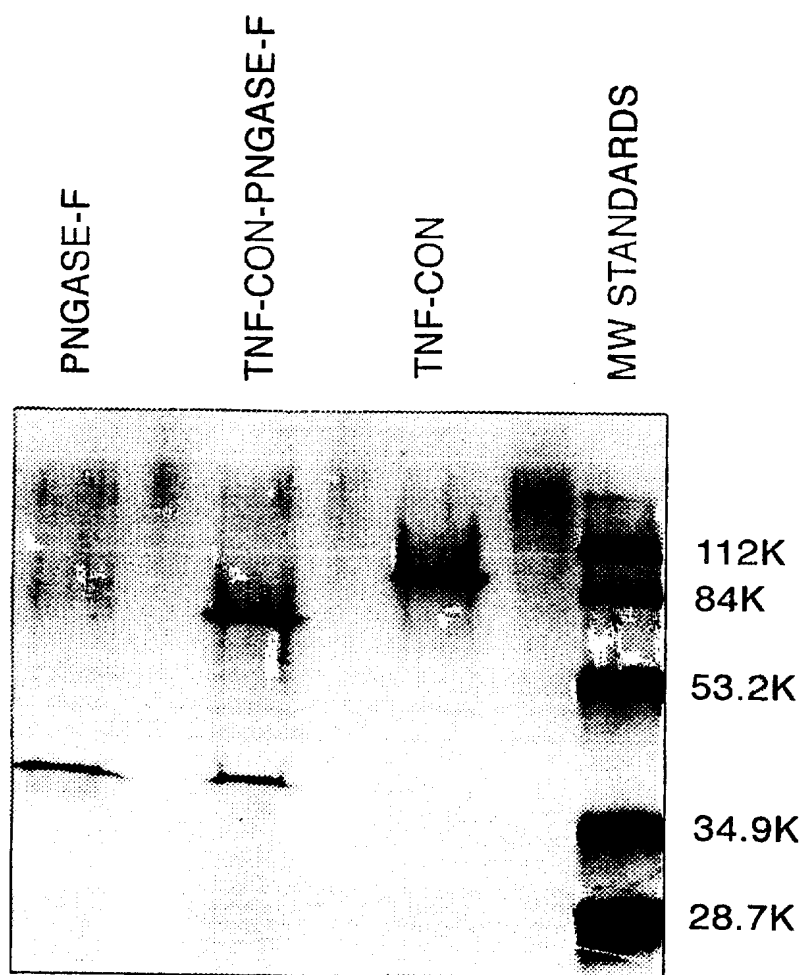
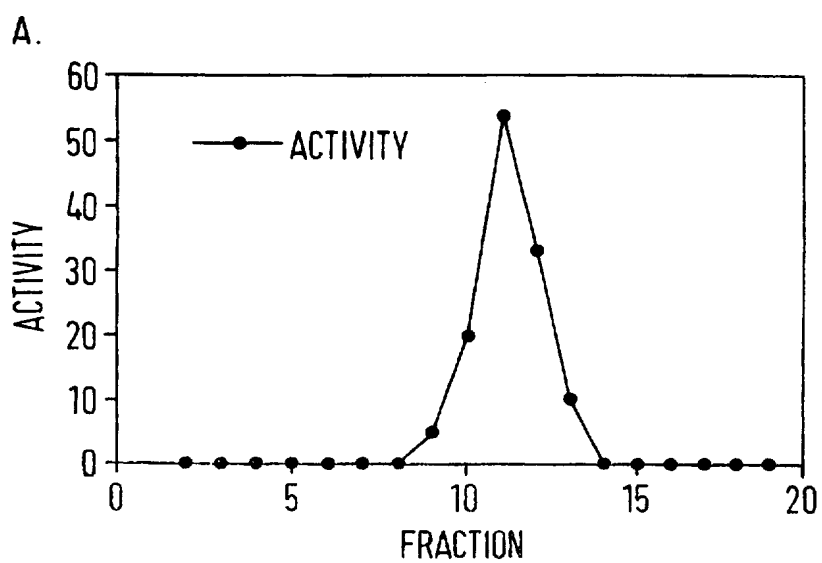


FIG. 3

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B.



FIG. 4

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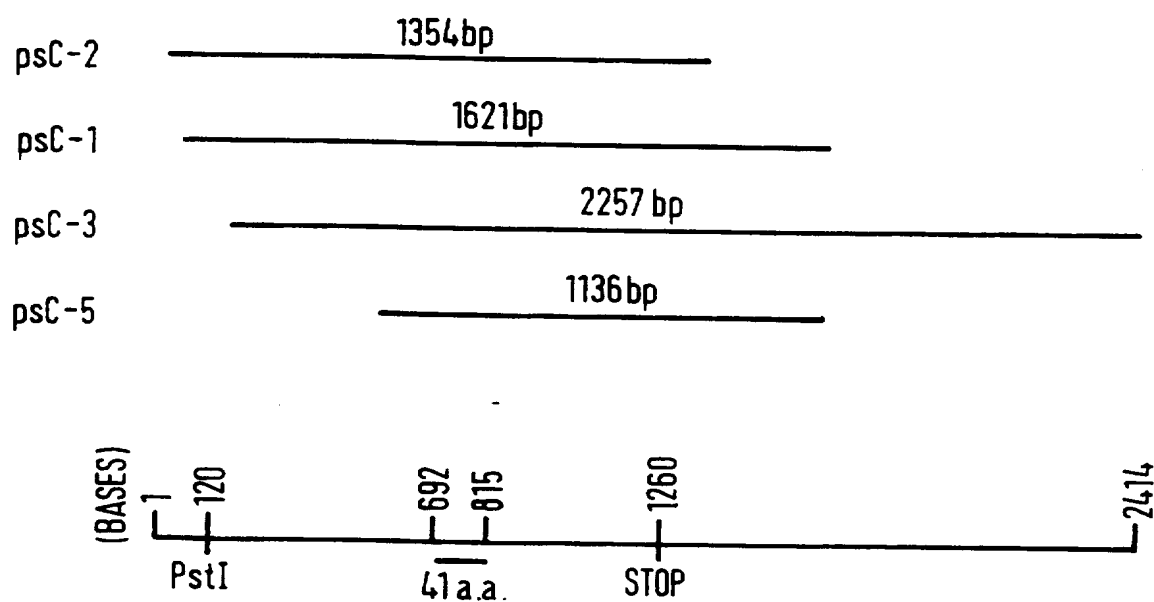


FIG. 5

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GAA GCT GAC CTT GTG ACA ACT CAT GAA TTG GGG CAC AAT TTT GGA GCA Glu Ala Asp Leu Val Thr His Glu Leu Gly His Asn Phe Gly Ala	48
GAA CAC GAT CCA GAT GGT TTA GCA GAA TGT GCC CCA AAC GAG GAC CAG Glu His Asp Pro Asp Gly Leu Ala Glu Cys Ala Pro Asn Glu Asp Gln	96
GGA GGA AAA TAC GTC ATG ATG TAT CCC ATA GCC GTG AGT GGT GAT CAT GAG Gly Gly Lys Tyr Val Met Tyr Pro Ile Ala Val Ser Gly Asp His Glu	144
AAC AAC AAG ATG TTT TCA AAC TGC AGT AAA CAG TCC ATC TAT AAG ACC Asn Asn Lys Met Phe Ser Asn Cys Ser Lys Gln Ser Ile Tyr Lys Thr	192
ATT GAA AGT AAG GCC CAG GAG TGT TTT CAA GAG CGC AGC AAC AAA GTG Ile Glu Ser Lys Ala Gln Glu Cys Phe Gln Glu Arg Ser Asn Lys Val	240
TGT GGC AAC TCC AGG GTG GAT GAG GGG GAG GAG TGC GAC CCC GGC ATC Cys Gly Asn Ser Arg Val Asp Glu Glu Gly Glu Cys Asp Pro Gly Ile	288
ATG TAC CTG AAC AAC GAC ACC TGC TGC AAC AGC GAC TGC ACC CTG AGG Met Tyr Leu Asn Asn Asp Thr Cys Cys Asn Ser Ser Asp Cys Thr Leu Arg	336
CCG GGC GTC CAG TGC AGT GAT AGG AAC AGT CCT TGC TGT AAA AAC TGT Pro Gly Val Gln Cys Ser Asp Arg Asn Ser Pro Cys Cys Lys Asn Cys	384
CAG TTC GAG ACG GCC CAG AAG AAG TGC CAG GAG GCT ATT AAT GCC ACT Gln Phe Glu Thr Ala Gln Lys Lys Cys Gln Glu Ala Ile Asn Ala Thr	432
TGC AAA GGC GTG TCT TAC TGC ACA GGT AAC AGC AGT GAG TGC CCC CCT Cys Lys Gly Val Ser Tyr Cys Thr Gly Asn Ser Ser Glu Cys Pro Pro	480
CCG GGA AAC GCC GAG GAC ACG GTG TGC CTG GAC CTG GGC AGG TGC Pro Gly Asn Ala Glu Asp Asp Thr Val Cys Leu Asp Leu Gly Arg Cys	528

FIG. 6(I)

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AAG GAC GGC AAG TGC GTG CCC TTC TGC GAG CGG GAG CAG CGG CTG GAG Lys Asp Gly Lys Cys Val Pro Phe Cys Glu Arg Glu Gln Arg Leu Glu	576
TCC TGC GCG TGT AAC GAA ACC GAC CAC TCG TGC AAG GTG TGC TGC CGG Ser Cys Ala Cys Asn Glu Thr Asp His Ser Cys Lys Val Cys Cys Arg	624
GCC CCC TCG GGC CGT TGG CTG CCC TAC GTG GAC GCC GAA CAG AAG AAC Ala Pro Ser Gly Arg Trp Leu Pro Tyr Val Asp Ala Glu Gln Lys Asn	672
TTG TTT TTG AGG AAG GGG AAG CCC TGT ACA GTA GGA TTT TGT GAC ATG Leu Phe Leu Arg Lys Gly Lys Pro Cys Thr Val Gly Phe Cys Asp Met	720
AAT GGC AAG TGT GAG AAG CGA GTG CAG GAC GTC ATC GAG CGG TTC TGG Asn Gly Lys Cys Glu Lys Arg Val Gln Asp Val Ile Glu Arg Phe Trp	768
GAG TTC ATT GAC AAG CTG AGC ATC AAT ACT TTC GGG AAG TTC CTG GCA Glu Phe Ile Asp Lys Leu Ser Ile Asn Thr Phe Gly Lys Phe Leu Ala	816
GAC AAC ATC GTG GGC TCC GTC CTG GTG TTC TCC CTG ATG CTC TGG ATC Asp Asn Ile Val Gly Ser Val Leu Val Phe Ser Leu Met Leu Trp Ile	864
CCC GTC AGC ATC CTC GTC CAC TGC GTG GAT AAG AAG CTG GAT AAG CAG Pro Val Ser Ile Leu Val His Cys Val Asp Lys Lys Leu Asp Lys Gln	912
TAC GAA TCC CTG TCT CTG CAC CCC AGC AAC GTG GAG ATG CTA AGC Tyr Glu Ser Leu Ser Leu Leu His Pro Ser Asn Val Glu Met Leu Ser	960
AGC ATG GAT TCA GCA TCC GTT CGC ATC ATC AAG CCC TTT CCT GCG CCC Ser Met Asp Ser Ala Ser Val Arg Ile Ile Lys Pro Phe Pro Ala Pro	1008

FIG. 6(II)

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CAG ACC CCA GGC CGC CTG CAG CCC CTG CAG CCC CTG CAG CCC GGC CCC Gln Thr Pro Gly Arg Leu Gln Pro Leu Gln Pro Leu Gln Pro Gly Pro	1056
GTG CTG CCC TCT GCG CCT TCG GTG CCC GTG GCT CCA AAA CTG GAC CAC Val Leu Pro Ser Ala Pro Ser Val Pro Val Ala Pro Lys Leu Asp His	1104
CAG CGG ATG GAC ACC ATC CAG GAG GAC CCC AGC ACG GAC TCG CAC GTG Gln Arg Met Asp Thr Ile Gln Glu Asp Pro Ser Thr Asp Ser His Val	1152
GAC GAG GAC GGC TTC GAG AAG GAC CCT TTC CCC AAC AGC AGT GCC GCT Asp Glu Asp Gly Phe Glu Lys Asp Pro Phe Pro Asn Ser Ser Ala Ala	1200
GCC AAG TCA TTT GAG GAT CTC ACG GAC CAT CCG GTC ACG AGA AGT GAA Ala Lys Ser Phe Glu Asp Leu Thr Asp His Pro Val Thr Arg Ser Glu	1248
AAG GCC TCG TCC TTT AAG CTG CAG CGC CAG AGT CGC GTT GAC AGC AAG Lys Ala Ser Ser Phe Lys Leu Gln Arg Gln Ser Arg Val Asp Ser Lys	1296
GAA ACG GAG TGC TAGTTCAGGG CTGCGTCCGG AGCTCTCGGA CTTGGCGTGC Glu Thr Glu Cys ***	1348
AGGATATTTT TATAGATTG ACCTCAGATC ACTGCCGAGA TCTTGTGAAG ATTGGGAGA	1408
AAAAAGGAAG TGACTTCCCA GCAGATGCTG GTCATGAGTT TGGACTTCCT CCACCTCCGT	1468
GAGACTGTGT GGGTAGCCCT TTTCTTTGA AGAGGTAAGG CGAACCTAGC TTACTTTGAG	1528
GCCTTCAGGT TTTAGTCTTT TGATCTTTAA AATATCTTTC AACCTGTGGT GCAGAAGCAG	1588
AAACCACAGC TGGATTATGT TATGACTATT TACGTTTTTG TAAATTACCT TTATATTGAG	1648

FIG. 6(III)

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AACAGCACTG ATTGTGGAGG TGATCATGTT TTTAAGACAC TGTAACGATC CAATGAACAC 1708
AAAGAGGCAT TTCATCAGTT TCCTGCGAGG ATAAC TGGA CACAGAAATAG ATGGATTCT 1768
TCCCTTCAGG TCAAATAAAG TCCGAGGTGC GTAATGTCAC CCAAATCTGC TTTCTGATGC 1828
AGAACCCGGT TCTACGTTCC CCCCAAAGGT GGGTTCAGGT TCGAGGTATT GCCTAGAACT 1888
TAGAGCAGAA GGGAGACCAC ACATCTGAAT TTAGGAATAA ACTGGGTAA TGTTCCTCC 1948
TGTTTCTAAA TG TTCAGAAAT TTCTAATTTC TAGAATTAAA ACAATCTAGC TTTATAGTAA 2008
AGATTATAAA TTATAGCCAC ATGAATTGAC AGAATCCTGC TGATTAACTG TTTAGGGCCA 2068
TACCTAGCTG GGCAGTTTTT CTGGGTTTTG TAAGTATCTT CCTGTCTCTC TACGGGTGCC 2128
ACGCTCCTAA AAAACCCACC ATCCACAAA CCTCAACCCT TCAGAACAGG AGGACGGTCG 2188
CCTCTTCGCA AACACTAGTG GCCCAGACGG TCGGTGCGGT TGGGCGCGCT TGCCAGGCGA 2248
ACAGGGTGCG AGCGGTGGC CGGGGGTCTG TCCTCAGACC ATTGGTCCCT AGACCCCTAGA 2308
CCTTCTGGCT CGCGTGCTTC GGCACCGTCT GCGCGCCCTG CGGAGGGAT AAAAACCATC 2368
CGGCTGAGTT CTGGTTTCAA AAGCTTTTTC CCTGCTAAG GACTGCAGTG TGCTCACCAG 2428
CCAGCACCCC TTAACAGGTA ACTTGTAAGC TTCGAG 2464

FIG. 6(IV)

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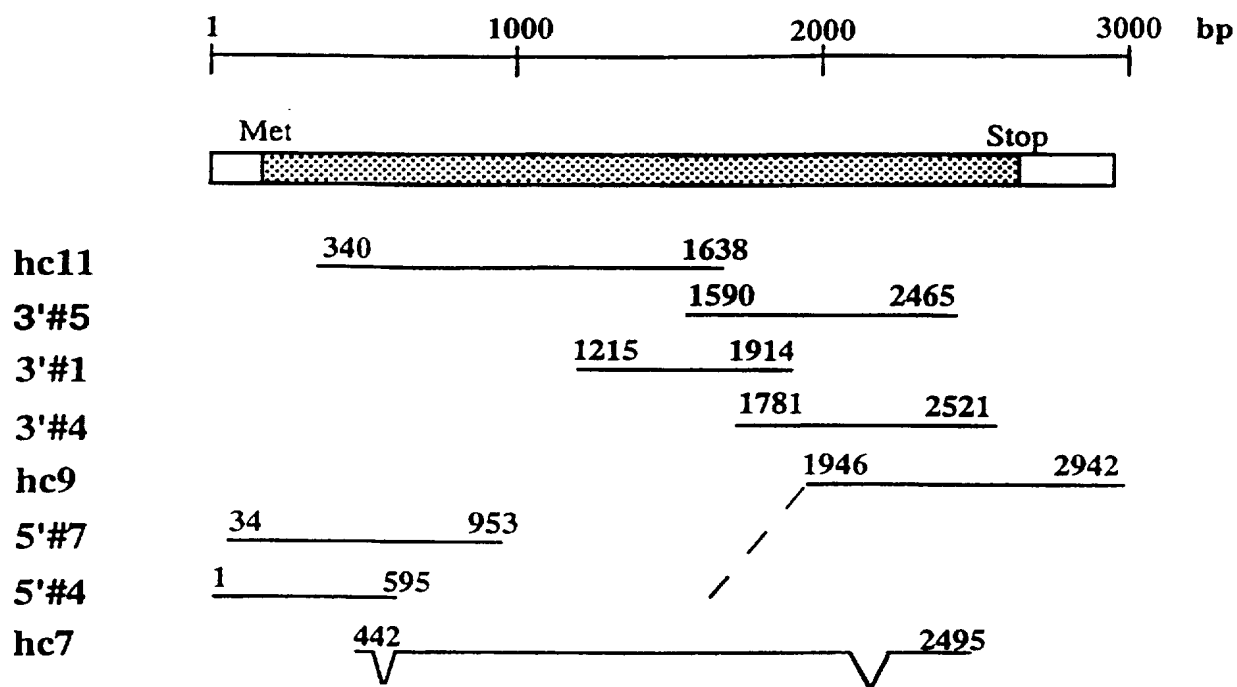


FIG. 7

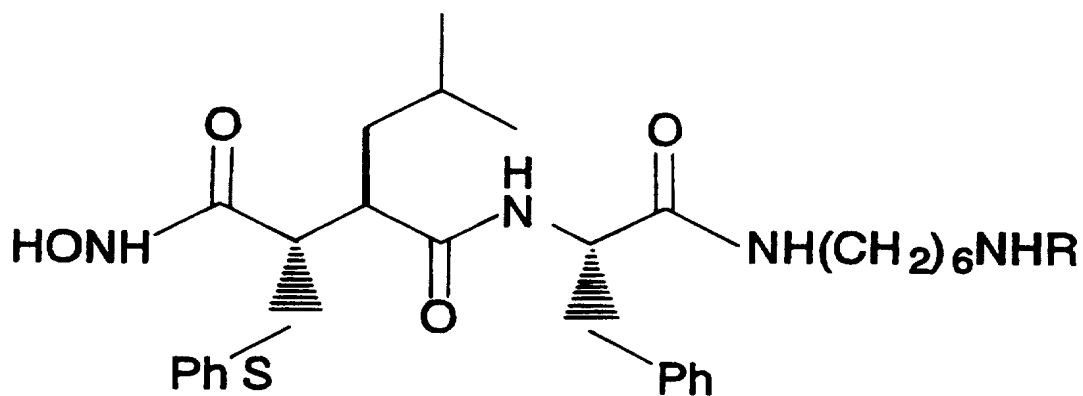


FIG. 8

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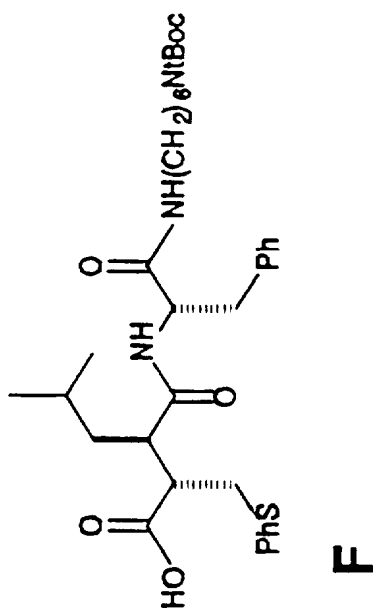
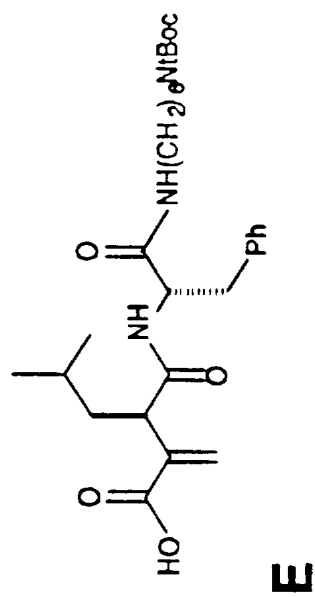
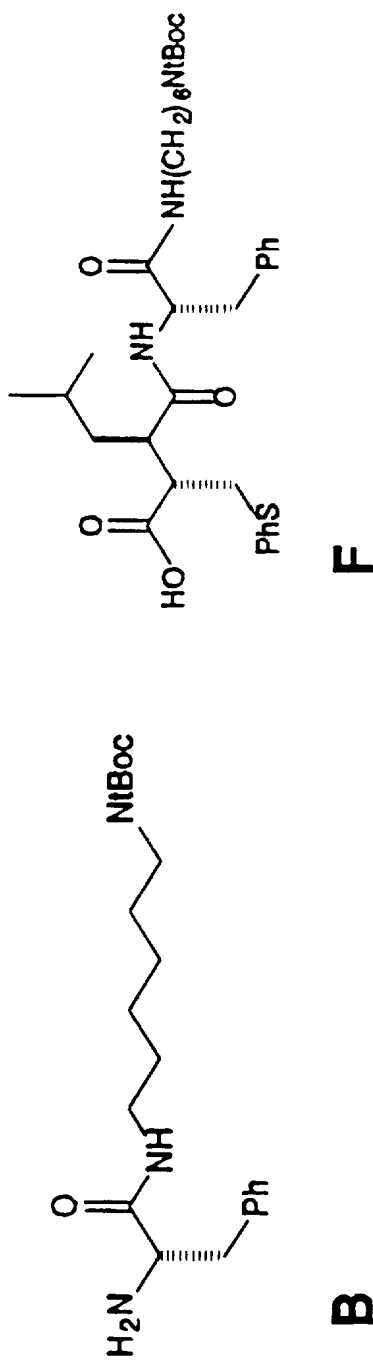
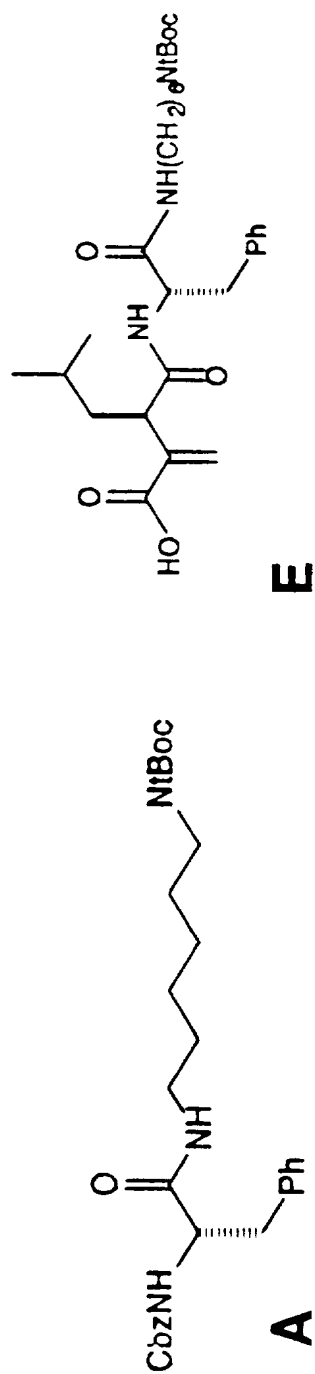


FIG. 9(I)

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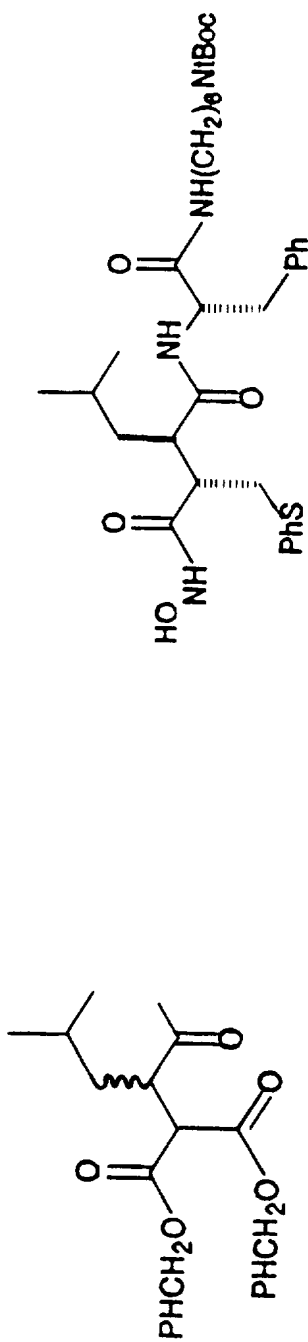
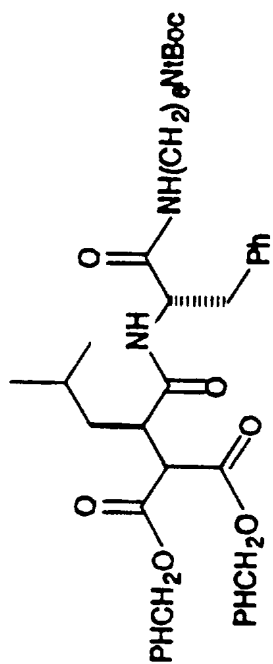
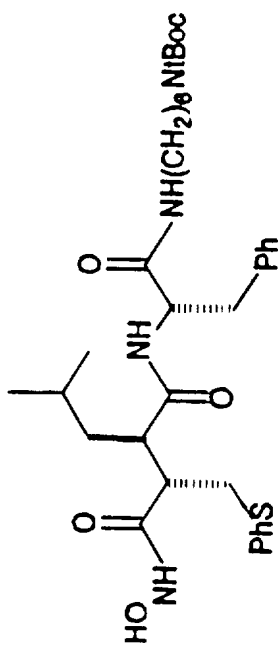
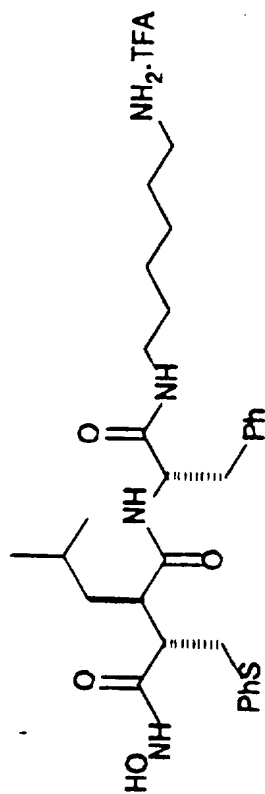
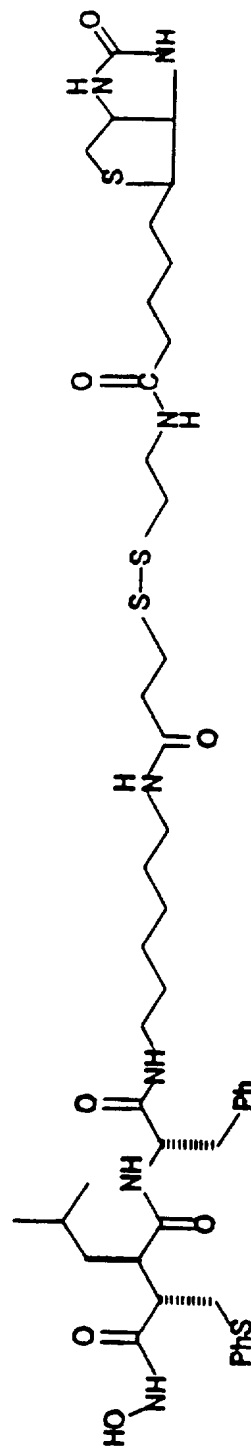
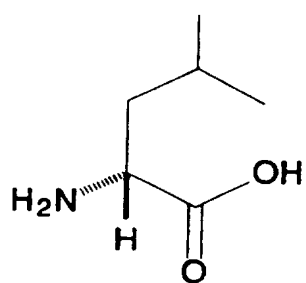
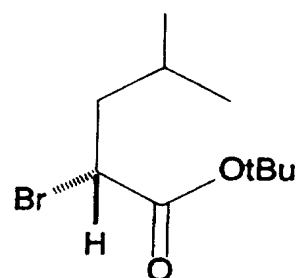
**G****D****H**

FIG. 9(II)

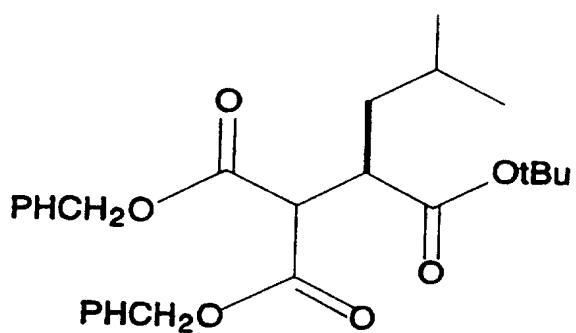
19 / 20



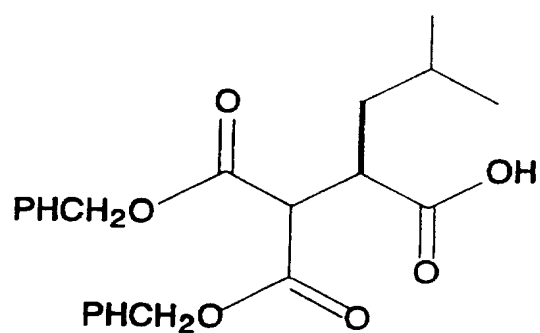
A



B



C



D

FIG. 10

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AAACTGGCTG AACCGGCCTT TTGGTAACGC CACCTTCACT TCTGGGGGCG TCGAACCTGG 60

CGGTAGAATC TTCCCAGTAG GCGGCGCGGG AGGAAAAGA GGATTGAGG GCTAGGCCGG 120

GCGGATCCCG TCCTCCCCCG ATGTGAGCAG TTTTCCGAAA CCCCCTCAGG CGAAGGCTGC 180

CCAGAGAGGT GGAGTCGGTA GCGGGGCGCG GAACATGAGG CAGTCTCTCC TATTCCTGAC 240

CAGCGTGGTT CCTTTCGTGC TGGCGCGCGG ACCTCCGGAT GACCCCGGCT TCGGCCCCCA 300

CCAGAGACTC GAGGAGCTTG ATTCTTTGCT CTCAGACTAC GATATTCTCT CTTTATCTAA 360

TATCCAGCAG CATTCGGTAA GAAAAAGAGA TCTACAGACT TCAACACATG TAGAAACACT 420

ACTAACTTTT TCAGCTTTGA AAAGGCATTT TAAATTATAC CTGACATCAA GTACTGAACG 480

TTTTTCACAA AATTTCAGG TCGTGGTGGT GGATGGTA 518

FIG. 11